Pathogenesis of hantavirus infection in the endothelial cell model.

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Come on. Let's keep a little optimism here!

Han Solo, The return of the Jedi (1983)

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List of abbreviations

APC	antigen presenting cell
APS	ammonium persulfate
BSA	bovine serum albumin
BSL	biosafety level
CD	cluster of differentiation
CPE	cytopathic effect
Cy5.5	(a cyanine dye with absorbance maximum at 675 nm and emission maximum at 694 nm)
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium (for cell culture)
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FACS©	fluorescence associated cell sorter
FCS	fetal calf serum
FFU	focus forming unit
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCPS	hantavirus cardiopulmonary syndrome
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HFRS	hemorrhagic fever with renal syndrome
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IL	interleukin
IRDye™800	(an infrared dye with absorbance maximum at 778 nm and emission maximum at 806 nm)

IRF	interferon regulatory factors
ISRE	interferon stimulated response element
kDa	kiloDalton
KHF	Korean hemorrhagic fever
LPS	lipopolysaccharide
MAP kinase	mitogen-associated protein kinase
MFI	mean fluorescence intensity
MOI	multiplicity of infection
N	nucleocapsid
NE	nephropathia epidemica
NK	natural killer
OAS	2'-5'-oligoadenylate synthetase
OD	optical density
PAMP	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
PFA	paraformaldehyde
PMBC	peripheral blood mononuclear cells
PKR	protein kinase R
poly I:C	polyinosinic-polycytidylic acid
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium (for cell culture)
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STAT	signal transducer of activation and transcription
TAP	transporter associated with antigen
TGF	transforming growth factor
TNFα	tumor necrosis factor α
Tris-HCl	Tris-(hydroxymethyl)-aminomethan
TUNEL	terminal-desoxynucleotidyl-transferase-mediated dUTP nick end labeling
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule-1

Viruses

HTNV	Hantaan virus
TULV	Tula virus
PUUV	Puumala virus
DOBV	Dobrava virus
SEOV	Seoul virus
PHV	Prospekt Hill virus
SNV	Sin Nombre virus
EBV	Epstein Barr virus
CMV	Cytomegalovirus
ECMV	encephalomyocarditis virus
LCMV	lymphocytic choriomeningitis virus

Summary

Hantaviruses represent important human pathogens and can induce hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Both diseases are characterised by endothelial dysfunction. Although hantaviruses replicate in human endothelial cells they do not cause any apparent cytopathic effect. This major paradox suggests that immunopathological mechanisms might be involved in pathogenesis.

Activation of the endothelium might play a major role in the pathophysiology of inflammatory processes eventually leading to endothelial dysfunction. Therefore, the complex series of cellular events which occurs during inflammation was investigated in hantavirus-infected human umbilical vein endothelial cells (HUVEC). The capacity of pathogenic and rather nonpathogenic hantaviruses to induce an antiviral response in HUVEC was compared. To develop a concept of hantavirus immunopathogenesis, the role of immune cells in the course of an infection was evaluated.

However, the potential risk of accidental infection by hantaviruses in the research laboratory necessitates special precautionary measures. Certain safety prerequisites, including appropriate virus inactivation procedures to permit necessary further processing of specimens outside of the biosafety level 3 laboratory, had to be achieved prior to taking up the experimental part of this study. Virus inactivation and depletion methods described in this study were shown to be suitable to prepare non-infectious samples for further use in immunological, virological and cell biological assays. With this knowledge and the appropriate safety precautions the systematic experiments aimed at discovering pathogenic mechanisms of hantavirus disease could be launched.

Infection of endothelial cells with both Hantaan virus (HTNV) or Tula virus (TULV) did not induce activation of the highly conserved cytosolic serine/threonine protein kinases p38 or p42/44. Moreover, it did not lead to activation of central transcription factor NFκB. Likewise, a modulation of its inhibitor IκB-α could not be observed after infection. Finally, the results did not reveal significantly increased expression levels of cell adhesion molecules VCAM-1 or ICAM-1 in response to an infection with HTNV or TULV. Thus, both hantaviruses presumably do not belong to

the stress factors known to activate signalling events involved in inflammatory responses.

The innate immune response triggered by HTNV and TULV was compared in endothelial cells. Both HTNV- and TULV-infected cells showed increased levels of molecules involved in antigen presentation. However, TULV-infected HUVEC more rapidly upregulated HLA class I molecules. Interestingly, HTNV clearly induced the production of interferon-β (IFN-β) whereas expression of this cytokine was barely detectable in the supernatant or in extracts from TULV-infected HUVEC. Nevertheless, the upregulation of HLA class I on both TULV- and HTNV-infected cells could be blocked by neutralising anti-IFN-β antibodies. Most strikingly, the antiviral MxA protein, which interferes with hantavirus replication, was induced already 16 h after infection with TULV. In contrast, HTNV-infected HUVEC showed no expression of MxA until 48 h postinfection. In accordance with the kinetic of MxA expression TULV only inefficiently replicated in HUVEC whereas HTNV-infected cells produced high titers of virus particles that decreased after 48 h postinfection. Both hantavirus species, however, could replicate equally well in Vero E6 cells which lack an IFN-induced MxA response. Taken together, the results obtained suggest that a delayed induction of antiviral MxA in endothelial cells after infection with HTNV could allow viral dissemination and contribute to the pathogenesis leading to HFRS or HCPS.

Zusammenfassung

Hantaviren stellen wichtige menschliche Krankheitserreger dar und können das Hämorrhagische Fieber mit renalem Syndrom (HFRS) sowie das Hantavirus kardiopulmonale Syndrom (HCPS) hervorrufen. Beide Erkrankungen sind durch endotheliale Funktionsstörungen charakterisiert. Obwohl sich Hantaviren in humanen Endothelzellen replizieren, verursachen sie dabei keine offensichtlichen zytopathischen Effekte. Dieses Paradoxon führt zu der Annahme, dass immunpathologische Mechanismen eine bedeutende Rolle in der Pathogenese dieser Erkrankung spielen könnten.

Die Aktivierung des Endothels, die schließlich zu endothelialen Funktionsstörungen führt, ist für die Pathophysiologie entzündlicher Prozesse von entscheidender Bedeutung. Folglich wurden die komplexen zellulären Ereignisse, die im Verlauf einer Entzündung auftreten, in Hantavirus-infizierten humanen Nabelschnur-Endothelzellen (HUVEC) untersucht. Die Fähigkeit pathogener Hantaviren, eine antivirale Antwort in infizierten Endothelzellen auszulösen, wurde mit der weniger pathogener Hantaviren verglichen. Mit dem Ziel, ein Konzept zur Immunpathogenese bei einer Hantavirusinfektion zu entwickeln, wurde zudem die Rolle von Immunzellen im Verlauf einer solchen Infektion untersucht.

Das Risiko, sich während der Arbeit im Forschungslabor versehentlich mit Hantaviren zu infizieren, macht spezielle Sicherheitsmaßnahmen zwingend erforderlich. Solche Richtlinien zum sicheren Umgang mit dem Virus selber mussten erarbeitet werden, bevor mit dem experimentellen Teil der Arbeit begonnen werden konnte. Erst dann war nämlich eine weitere Bearbeitung der Proben auch ausserhalb des Hochsicherheitslabors möglich. Die hier beschriebenen Maßnahmen zur Desinfizierung von infizierten Zellen und deren Zellkulturüberständen sind geeignet, eine sichere Handhabung von Proben in immunologischen, virologischen sowie zellbiologischen Untersuchungen zu gewährleisten. Mit dieser Gewissheit und unter Einhaltung der Vorsichtsmaßnahmen konnte schließlich mit den Untersuchungen zu den pathogenen Mechanismen der durch Hantaviren hervorgerufenen Erkrankungen begonnen werden.

Eine Aktivierung der hochkonservierten zytosolischen Serin/Threonin Proteinkinasen p38 und p42/44 konnte nach einer Infektion mit dem pathogenen Hantaan Virus (HTNV) oder dem weniger pathogenen Tula Virus (TULV) nicht

nachgewiesen werden. Eine Aktivierung des zentralen Transkriptionsfakors NFκB wurde darüber hinaus ebenso wenig beobachtet wie die Degradierung seines zytoplasmatischen Inhibitors IκB-α. Des Weiteren konnte auch keine eindeutig erhöhte Expression der Zelladhäsionsmolekülen ICAM-1 oder VCAM-1 auf der Oberfläche von HTNV- oder TULV-infizierten Endothelzellen festgestellt werden. Demnach gehören sowohl die pathogenen wie auch die weniger pathogenen Hantaviren vermutlich nicht zu den auslösenden Faktoren von Signalkaskaden, die grundsätzlich im Zusammenhang mit entzündlichen Prozessen stehen.

Die durch eine Infektion ausgelöste angeborene Immunantwort der HUVEC wurde untersucht, wobei insbesondere die Reaktionen auf eine Infektion mit HTNV oder TULV miteinander verglichen wurden. Tatsächlich konnte sowohl in HTNV- wie auch in TULV-infizierten HUVEC eine deutlich gesteigerte Expression von Antigen-präsentierenden Molekülen beobachtet werden. Dabei war das weniger pathogene TULV sogar noch effizienter in der Lage, die Expression der Klasse I HLA-Moleküle zu induzieren. Interessanterweise löste dagegen eine Infektion mit dem pathogenen HTNV die Freisetzung von Interferon (IFN)-β in den Zellkulturüberstand aus, wohingegen das bei TULV-infizierten Endothelzellen kaum zu beobachten war. Nichtsdestotrotz konnte die Induktion der Klasse I HLA-Moleküle in beiden Fällen durch neutralisierende Antikörper gegen IFN-β blockiert werden. Das antiviral wirksame MxA-Protein jedoch, welches die Replikation der Hantaviren behindert, konnte in TULV-infizierten Endothelzellen bereits 16 Stunden nach der Infektion nachgewiesen werden, was bei HTNV-infizierten Zellen erst nach 48 Stunden gelang. Dementsprechend replizierte sich das TULV deutlich weniger effizient als das HTNV in humanen Endothelzellen, wobei allerdings nach zwei Tagen der Infektion ein deutlicher Titerabfall auch hier zu beobachten war. Beide Hantaviren waren jedoch gleichermaßen in der Lage, sich in Vero-Zellen zu replizieren, denen eine IFN-induzierte MxA-Antwort fehlt. Insgesamt legen die hier gezeigten Ergebnisse nahe, dass die verspätete Induktion des antiviral wirksamen MxA-Proteins in HTNV-infizierten HUVEC dazu führt, dass sich das Virus erfolgreich im Organismus verbreiten kann und damit zur Pathogenese des HFRS oder HCPS beiträgt.

1 Introduction

1.1 Hantaviruses

1.1.1 Historical context

The Korean War in the 1950s was of pivotal importance for the recognition of hantaviruses: More than 3000 soldiers from the United Nations fell ill with Korean hemorrhagic fever (KHF). This febrile illness with renal failure and shock, frequently accompanied by hemorrhagic manifestations, was associated with a case fatality rate of about 5 to 10 % [Smadel J.E., 53]. The outbreak attracted world attention. Despite intensive research the origin of this disease remained obscure. However, it became clear that this was not a new disease and it was recognised that there were close similarities to the long known hemorrhagic fever with renal syndrome (HFRS). It was not until 1978, though, when a new virus was detected in the lungs of infected *Apodemus agrarius* using convalescent sera from KHF patients. Hantaan virus, named after the Hantaan river in Korea, was successfully propagated in its rodent hosts and eventually isolated in cell culture in 1981 [French, 81; Lee, 78].

In 1993 an accumulation of cases of fatal respiratory distress of unknown origin was documented in the Four Corners region in the USA. Unexpectedly, sera from patients reacted with hantaviral antigen [Nichol, 93]. These findings boostered the research efforts and eventually led to the identification of several new hantaviruses [Ksiazek, 95; Schmaljohn, 95] as causative agents of the hantavirus cardiopulmonary syndrome (HCPS).

Currently, these two human diseases are associated with a hantavirus infection: HFRS together with its milder form referred as nephropathia epidemica (NE) and HCPS.

1.1.2 Virology

Taxonomic status and genetic organisation.

Several of the most important human pathogens in the family of *Bunyaviridae* are included within the separate genus *Hantavirus*. Like all members of this family

hantaviruses have a segmented RNA genome. All three negative sense, single-stranded segments encode only one protein, although minor open reading frames have been identified [Jonsson, 01]. The large (L) segment encodes the viral polymerase, whereas the medium (M) segment codes for a polyprotein cleaved cotranslationally into two surface glycoproteins, termed G1 and G2. The nucleo (N) protein, forming the nucleocapsid, is encoded by the small (S) segment [Schmaljohn, 96]. Hantaviruses carried by *Arvicolinae* and *Sigmodontinae* rodents, but not those carried by *Murinae* rodents, possess an open reading frame of not yet clearly defined functionality in their S segment [Bowen, 95; Plyusnin, 94]. On the basis of the nucleotide sequences, mainly of the M and S segments, hantaviruses can be serologically classified. In addition, the focus reduction neutralisation test (FRNT) provides an efficient means for the serotyping of hantavirus-specific neutralising antibodies in infected persons [Heider, 01].

At least 30 hantaviruses have been found [Hart, 99; Kanerva, 98]. Only about half of these viruses have been isolated in cell culture so far, others have been identified by their genetic sequences.

Morphology.

By electron microscopy, hantaviruses appear to be spherical in shape with an average diameter of about 100 nm. The granulofilamentous interior composed of ribonucleocapsids is surrounded by a lipid bilayered membrane. Two glycoproteins, designated G1 and G2, are incorporated within the lipid envelope [Gonzalez-Scarano, 96].

The morphogenesis of hantaviruses has not yet been clearly defined. The virus assembly is thought to begin with the cotranslational cleavage of the polyprotein encoded by the M segment into two glycoproteins. Following glycosylation and folding of G1 and G2 inside the Golgi complex, the proteins are retained and gradually accumulate. The N protein forming the nucleocapsid is expressed in the cytoplasm. In fact, hantaviral antigen is generally not detected on the surface of an infected cell but is located in the perinuclear region. Together with the viral RNA segments the nucleocapsid is targeted to the Golgi complex, probably by specific recognition of the cytoplasmic part of the glycoproteins, where virus particles finally budd into the Golgi cisternae [Ravkov, 01].

Natural reservoir hosts.

Hantaviruses are usually closely associated with a particular reservoir host (Figure 1). The geographic distribution of the host determines the distribution of the virus and, accordingly, of human disease. Serological and virological studies, i.e. cell culture or genome detection, have shown evidence of hantaviruses in rodents almost worldwide with emphasis on Europe, the Americas and Asia [Schmaljohn, 97; Lee, 89].

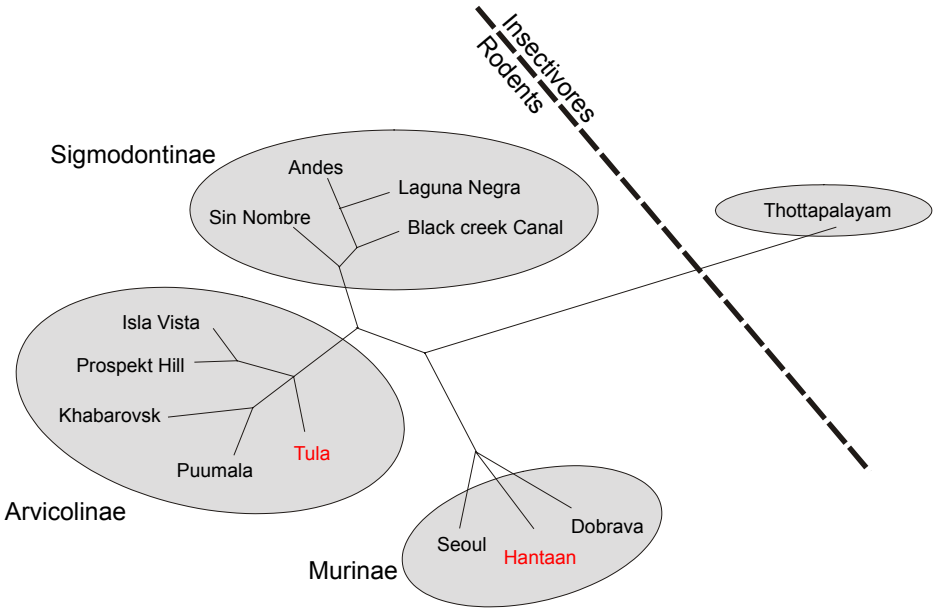

Figure 1. Phylogenetic relationship of hantaviruses.

Illustration of the parallel phylogeny between hantaviruses and their rodent hosts.The subfamilies of hantaviral rodent hosts (*Murinae*, *Arvicolinae* and *Sigmodontinae*) and the insectivore host are designated by the shaded regions. Genetically related rodents carry genetically similar hantaviruses. Positions and lengths of lines do not imply precise phylogenetic relationships. After: McCaughey and Hart, 2000 (modified).

Roughly, hantaviruses are subdivided into New World and Old World hantaviruses. Hantavirus species originating from New World rodents of the subfamily *Sigmodontinae* (New World rats and mice) are associated with HCPS. SinNombre (SNV) carried by *Peromyscus maniculatus*, a *Sigmodontinae* rodent, was responsible for the initial outbreak in the Four Corners area. Rodents from the subfamily *Murinae* (Old World rats and mice) carry hantaviruses which are associated with HFRS. Dobrava virus (DOBV), Hantaan virus (HTNV), or Seoul virus (SEOV), well-studied representatives of this genetic group, are found primarily in the Balkan region and in East Asia. Viruses hosted by the subfamily *Arvicolinae* (Old World voles) including Puumala (PUUV) are associated with NE, the milder form of

HFRS. Though Tula (TULV) and Prospekt Hill (PHV), viruses generally accepted to be rather nonpathogenic [Geimonen, 02; Schultze, 02; Yanagihara, 90], are both carried by *Arvicolinae* rodents, their distribution differs greatly. While *Microtus arvalis*, carrier of TULV, is found in Europe, Prospect Hill and its carrier *Microtus pennsylvaticus* are found in North America only [McCaughey, 00; Schmaljohn, 97].

Hantavirus infection in rodent hosts is asymptomatic and often long-lasting. Infectious virus is excreted with urine and other excreta for months or even years [Bernshtein, 99; Kanerva, 98]. During persistent infection in adult rodents viral antigen can be detected in endothelial cells and in many tissues including lung and kidney, heart and spleen [Botten, 00; Green, 98]. However, the presence of hantavirus in an individual rodent host does not provoke survival disadvantage.

In the Eurasian region human infections with hantaviruses are associated with a broad range of clinical presentations ranging from very severe, even lethal cases of HFRS after an infection with HTNV to mostly asymptomatic TULV infections.

Transmission.

Hantaviruses, unlike viruses of other bunyavirus genera, are not transmitted by arthropod vectors. Instead, they persistently infect rodents of the family *Muridae* (order *Rodentia*). Transmission among rodents and to humans occurs generally via the respiratory route by inhalation of aerosols from rodent excreta [Kanerva, 98], although infection has been transmitted by rodent bites as well [Gonzalez, 84; Hart, 99]. Therefore, the respiratory tract is probably the primary site of replication for hantaviruses both in rodent host and humans [McCaughey, 00]. Transmission to man is a dead end for the virus since humans do not secrete hantaviruses in large amounts during infection [Clement, 03]. Moreover, person-to-person transmission has so far been documented in individual cases only [Padula, 98; Pinna, 04; Wells, 97].

1.1.3 Infection in man

For most hantavirus infections in humans, asymptomatic or mild infections with unspecific manifestations outnumber the infections with severe symptoms. An estimation of the number of cases is therefore difficult. However, about 60 000 to 100 000 hospitalised cases are reported annually world-wide [Krüger, 01; Vapalahti, 03].

Clinical features.

The clinical presentation of human hantavirus disease varies with the virus, disease type (HFRS or HCPS) and probably with human host characteristics as well. Accordingly, case fatality rates range from 0.1% in NE, up to 15 % in HFRS and 30 to 50 % in HCPS [Lee, 96; Schmaljohn, 97]. Namely for nephropathia epidemica (NE) caused by infection with PUUV host factors affecting disease severity have been described. Patients with HLA-DRB1*0301 and HLA-B8 alleles are associated with severe NE, whereas those with HLA-B27 show a milder course of disease [Plyusnin, 97].

The most severe form of HFRS is caused by Hantaan virus in Asia and Dobrava virus in East Europe. It is characterised by an acute triad of fever, haemorrhage and renal failure. In severe cases capillary permeability and vascular leakage lead to shock and cardiovascular collapse [Tkachenko, 91].

HCPS has a very different presentation from HFRS, although laboratory findings are basically similar to those in HFRS [Zaki, 95]. Renal involvement is rare and haemorrhagic manifestations are uncommon. In addition to severe respiratory insufficiency caused by pulmonary oedema, shock and myocardial dysfunction also account for the high case fatality of HCPS [Nichol, 96].

However, clinical features of HFRS and HCPS each run across spectra of presentation and severity. There can be pulmonary involvment in NE [Kanerva, 96b] and HFRS-like symptoms in HCPS [Hjelle, 96]. The pathogenic potential of some hantaviruses is not clear at present [McCaughey, 00; Schultze, 02]. While PHV or TULV are usually considered to be nonpathogenic to humans, a recent report could clearly associate a case of HFRS with pulmonary involvement with TULV infection, which should therefore be considered as a hantavirus with potential pathogenicity for humans [Klempa, 03].

Pathological findings in humans.

The endothelium represents a primary target for hantavirus infections. Endothelial cells, however, do not seem to be morphologically damaged in HCPS or HFRS patients [Pensiero, 92; Yanagihara, 90; Zaki, 95]. Still, capillary leakage is a common feature for both diseases. In all organs the inflammatory response seems rather mild and only slight mononuclear cell infiltrates are seen [Kanerva, 98]. The mechanisms ultimately causing increased endothelial permeability in hantavirus

infections are still unknown. However, subtle morphological changes might disturb the function and integrity of endothelial cells lining the capillaries, rather than gross damage induced by the virus directly. According to this, immunological and chemical mediators of inflammation should be considered to play a major role in hantavirus pathogenesis [Cosgriff, 91; Zaki, 95].

1.1.4 Infection in cell culture

Hantaviruses infect a broad range of different cell types and generate persistent infections in most cell cultures including continuous cell lines like A549 (lung carcinoma) or primary endothelial cells and monocytes/macrophages as well as Vero cells [Kitamura, 83; Nagai, 85; Pensiero, 92; Yanagihara, 90]. Without causing visible cytopathic effects they replicate *in vitro* in the cytoplasm of the host cell and apparently do not disturb the protein synthesis. In this regard, differences between pathogenic and nonpathogenic hantaviruses are not obvious. It is therefore not clear how hantavirus infections cause disease [Kanerva, 98]. Since direct viral effects are not apparent in either case, indirect effects like cellular mediators released from infected cells should be considered as triggers for hantavirus pathogenesis. Moreover, interactions between target cells of a hantavirus infection like endothelial cells on the one hand and cells of the immune system on the other hand might be the clue to understanding hantavirus pathogenesis.

Generally, hantaviruses grow comparatively slowly and it takes several days to reach complete infection in cell culture. The delayed growth depends mainly upon the adaptation level of the virus to the cell type used [Temonen, 93; Yanagihara, 90]. Several blind passages are required to isolate virus from rodents or humans in cell culture during which the virus adapts to the cell culture. Still, isolation of infectious virus is often uneffective. In fact, only few cases of successful propagation of virus derived from humans have been reported so far [Avsic-Zupanc, 94; Galeno, 02; Juto, 97].

1.1.5 Safety aspects

Biosafety level (BSL) 3 is suitable for work with infectious agents which may cause serious or potentially lethal diseases as a result of inhalation [CDC, 99]. Therefore, in laboratory facilities all laboratory work involving the propagation of hantaviruses in cell culture should be conducted in BSL 3 conditions. Nevertheless, reports about laboratory-aquired hantavirus disease are well known throughout the

world. Human disease caused by contact with naturally or experimentally infected rodents as well as by hantaviruses present in continuous cell lines has been reported [Lloyd, 84; Lloyd, 86]. It is therefore of great importance not only to handle the virus according to its potential hazard but to use appropiate inactivation procedures if further analysis are required outside the BSL 3-laboratory. Like other lipid-enveloped viruses, hantaviruses are susceptible to most disinfectants like peracetic acid or active chlorine [Prince, 91]. However, these agents are usually not suitable for subsequent cell biological, virological and immunological investigations and have to be substituted.

1.2 Endothelial cells

Since hantaviruses are derived from aerosolised rodent excreta the respiratory tract is probably the primary site of infection and replication. Moreover, main symptoms in human disease are based upon vascular dysfunction. Therefore, endothelial cells have been postulated to be the main target in a hantavirus infection *in vivo* [Kanerva, 98]. In fact, viral antigen has been demonstrated in endothelial cells of capillaries and blood vessels of the lung during the early phase of disease [Zaki, 95].

1.2.1 Structure and function of the endothelium

The endothelium represents an important boundary layer between the lumen of blood vessel and tissues. As a single-cell layer it covers the surface of blood vessels and regulates exchanges between the bloodstream and the surrounding tissues. The endothelium is therefore involved in the structural and functional regulation of homeostasis and permeability [Short, 98].

Cytoskeleton.

For proper function of the endothelium its structural integrity is required and mainly ensured by cell-to-cell junctions between endothelial cells. Adherence junctions are necessary for the first contact between endothelial cells by mechanically holding the cells together. The main structures involved in the maintenance of the barrier function, however, are the tight junctions [Lum, 96], which are responsible for sealing adjacent cells. At these contact sites, transmembrane receptors like integrins are linked to the actin skeleton, anchoring the endothelial cells to the extracellular matrix and therewith maintaining endothelial integrity.

Changes in the morphology of endothelial cells such as the formation of stress fibers might be a first hint to an infection. The actin cytoskeleton is largely responsible for establishing the shape of the cell and for controlling the changes in shape that occur when a cell is exposed to stress stimuli, e.g. shear stress. Integrins function as transmembrane linkers which mediate bidirectional interactions between the extracellular matrix and the actin cytoskeleton. They also function as signal transducers, activating various intracellular signalling pathways, including the inositol phospholipid pathway, when activated. Moreover, chemical stimuli, like cytokines or inflammatory mediators, induce signalling pathways in endothelial cells to modulate vascular biology.

Integrins.

These heterodimeric receptors composed of α and β subunits mediate cell-cell adhesion, cell migration and recognition of the extracellular matrix [Hynes, 92b; Lampugnani, 91]. The cytoplasmic domains of integrins transduce signals from both the inside and outside of the cell and regulate the integrin-mediated signalling events [Liu, 00].

Integrins are abundant surface receptors of endothelial cells and platelets [Hynes, 92b]. They were recently found to bind receptors that regulate vascular permeability [Short, 98]. Integrins are also linked to intracellular cytoskeletal elements like actin, which play a role in the migration process on the extracellular matrix [Hynes, 92a; Lampugnani, 91]. Integrin-directed cellular migration is another important aspect of endothelial cell function in the maintenance of vascular integrity [Kevil, 98].

The cellular entry of pathogenic hantaviruses is facilitated by specific β_3 integrins, while nonpathogenic PHV enters the cell via β_1 integrins [Gavrilovskaya, 98; Gavrilovskaya, 99]. Both integrins are expressed on the surface of endothelial cells and macrophages, and hantaviruses are known to predominantly infect these cells [Gonzalez-Scarano, 96; Nolte, 95; Pensiero, 92]. The increased vascular permeability observed in hantavirus disease might be connected to the interaction of hantaviruses with integrins [Gavrilovskaya, 98; Gavrilovskaya, 99; Gavrilovskaya, 02].

Interactions of pathogenic hantaviruses with β_3 integrins are considered potential triggers of endothelial dysfunction and thereby contributing to increased vascular permeability observed in hantavirus disease.

1.2.2 Inflammatory pathways in the endothelium

The surface of the endothelium is exposed to all kinds of pathogens. Adhesion of pathogens to endothelial cells is often, albeit not necessarily, sufficient to activate the endothelium. This activation, however, plays a major role in the pathophysiology of inflammatory processes eventually leading to endothelial dysfunction.

The complex series of events which occurs during inflammation include vascular changes and cellular events. Enhanced vascular permeability and migration of leukocytes through vessel walls into adjacent tissues characterise inflammatory reactions. They are mediated by chemical substances such as cytokines, in particular interferons, initially derived from activated lymphocytes and monocytes. Endothelial cells, also activated during the early phase of the inflammatory response, show increased expression of adhesion molecules which facilitate adherence of inflammatory cells to the endothelium [Johnson-Leger, 03]. Subsequent signalling cascades within the endothelial cells eventually lead to the activation of the NFκB pathway resulting in the expression of proteins such as receptors required for immune recognition or proteins involved in antigen presentation as well as cytokines and chemokines [Hiscott, 01]. However, such inflammatory stimuli employed in the NFκB pathway induce yet another separate pathway eventually leading to the activation of mitogen-activated protein (MAP) kinases. This highly conserved family of kinases is one of the major mechanisms used by human cells to transduce extracellular signals into intracellular responses [Robinson, 97].

Cytokines.

Endothelial dysfunction is characterised by increased permeability caused either by direct endothelial infection or by rather indirect effects like the production of various cytokines by activated leukocytes, for instance. In response to these inflammatory mediators, second messenger molecules modulate the actin-based cytoskeletal system of the endothelial cells and eventually lead to the disruption of the endothelial barrier integrity. This modulation process involves phosphorylation of linkage proteins at the junction sites leading to the reorganisation of the actin skeleton [Lum, 96].

Proinflammatory cytokines such as interleukin (IL)-1α produced specifically at the site of infection mediate the activation of leukocyte integrins, while the ligands of these integrins, namely adhesion molecules Intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, are upregulated on the surface of the inflamed endothelium [De Caterina, 01]. Upon stimulation endothelial cells themselves are able to release cytokines that may further promote the inflammatory response thereby amplifying the host immune response. IL-6 is one such proinflammatory cytokine, produced by various cell types including monocytes, macrophages and endothelial cells. It interacts with its membrane receptor and a signaltransducing glycoprotein eventually resulting in activation of MAP kinases [Legendre, 03].

The endothelium is therefore considered both a source of and a target for inflammation [Hack, 01].

Interferons.

Amongst others the family of cytokines comprises interferons (IFN) which are mainly known for their antiviral activities against a wide spectrum of viruses. They are proteins produced by cells in response to virus infection and a number of other stimuli including nucleic acids, synthetic oligonucleotides (poly I:C) or bacterial lipopolysaccharides [Paludan, 00]. These cytokines upregulate the expression of antigen presenting molecules such as human leukocyte antigen (HLA) class I which are recognised by cytotoxic CD8-positive T lymphocytes thus eliminating virus-infected cells. On exposure to an IFN cells develop an antiviral state in which a network of genes encoding factors with direct antiviral activity is stimulated. IFN, however, also affect cell growth and cell function (Figure 2). Type-1 IFN are synthesised not only in response to virus infection but are also produced at basal levels in the absence of a viral infection [Taniguchi, 01].

The three main human IFN, can be subdivided into type-1 and type-2 IFN. IFN-α and IFN-β, so called type-1 IFN, are thought to bind to the same receptor on the cell surface of target cells. This receptor differs from that of IFN-γ representing type-2 IFN. Both types of receptors are expressed on nearly all cell types [Schreiber, 93].

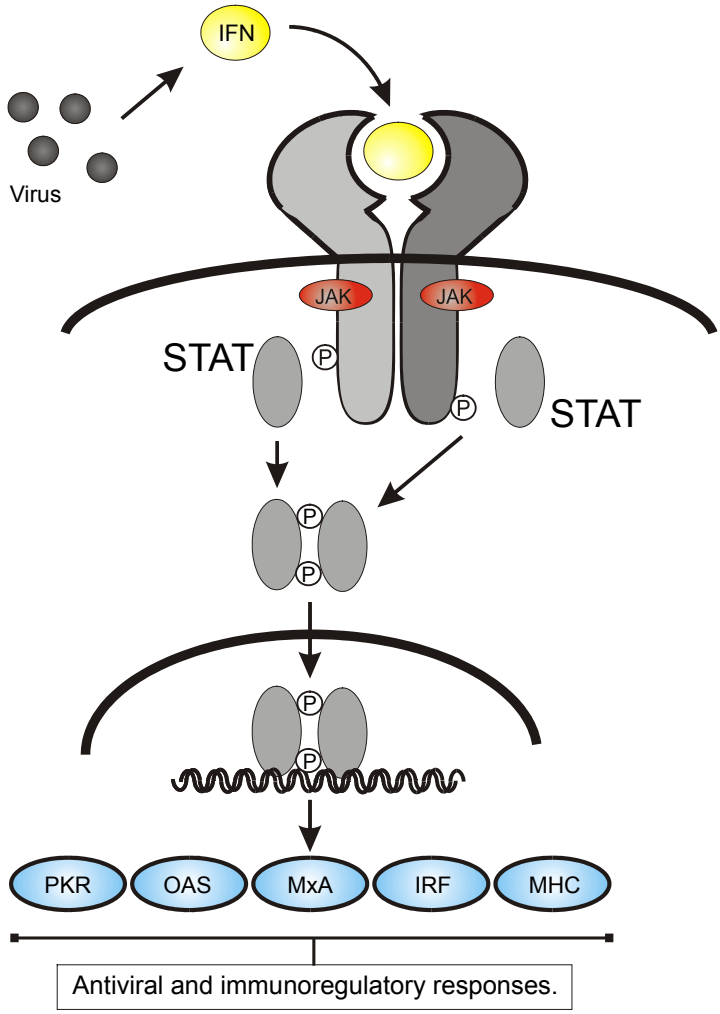

Figure 2. Molecular mechanisms of type 1 IFN-induced antiviral pathways.

Interferons (IFN) are ubiquitously expressed cytokines that interfere with virus replication within different cell types by activating a number of host genes and several parallel antiviral pathways. Two major intracellular actors of type-1 IFN-induced antiviral states are ribonucleic acid-dependent protein kinase (PKR) and 2'-5'-oligoadenylate synthetase (OAS), both being induced by type-1 IFN and activated by viral double stranded RNA. In addition, MxA proteins have also been implicated in type-1 IFN induced antiviral responses to some RNA viruses. Viruses, in turn, have evolved different strategies to escape control imposed by type-1 IFN and by type-1 IFN-induced antiviral factors. This includes the disruption of dsRNA and IFN receptor/Janus kinase (JAK) and signal transducer of activation and transcription (STAT) signalling events, the inhibition of interferon regulatory factors (IRF) and major histocompatibility complex (MHC) functions. The fatal outcome of virus infection is determined by complex interactions between viral virulence factors and cellular antiviral type-1 IFN-inducible factors. From: Katze et al., 2002 (modified).

IFN-α is produced by monocytes and macrophages, lymphoblastoid cells, fibroblasts, and a number of other cell types. IFN-β, a strictly species-specific IFN, is produced mainly by fibroblasts, epithelial and endothelial cells. It is involved in the regulation of unspecific humoral immune responses and immune responses against viral infections. IFN-β increases the expression of HLA class I molecules and blocks the expression of HLA class II molecules stimulated by IFN-γ [Lu, 95]. Additionally,

IFN-β stimulates the activity of natural killer cells (NK cells) and hence also antibody-dependent cytotoxicity. IFN-γ, at last, is an important immunomodulatory cytokine produced by activated T lymphocytes and NK cells, but not by endothelial cells.

Expression of IFN-α and IFN-β is controlled by the transcription factor NFκB together with Interferon regulatory factors (IRF) [Hiscott, 95]. Virus-induced posttranslational phosphorylation of IRF-3 is thought to stimulate IFN-β production. Secreted IFN-β then acts through the JAK-STAT pathway to stimulate the production of a another member of the IRF family, namely IRF-7, which in turn contributes to the transcriptional induction of different IFN-genes [Hiscott, 99; Marie, 98].

However, this complex interplay between the endothelium and the immune system during an inflammatory response or after a viral infection is still unexplained to a considerable extent.

Adhesion molecules.

Pathogens may use endothelial signalling pathways by binding to cell surface receptors and alter endothelial function by affecting endothelial monolayer integrity. These interactions are triggered by the characteristic properties of endothelial cells such as the expression of cell surface molecules, namely of the selectin and ICAM family.

Exposure to proinflammatory cytokines results in a massive increase in adhesion molecule expression on the surface of endothelial cells. Transmembrane glycoproteins of the selectin family, namely E-selectin, mediate the very first steps of leukocyte adhesion to the endothelium. In fact, *in vitro* induced upregulation of E-selectin molecules is transient and peaks already at six hours after stimulation, returning to baseline after 24 hours [Kuhns, 95]. E-selectin usually acts as signalling molecule for cell-cell interactions in the processes of vascular permeability and inflammation.

While E-selectin is unique to the endothelium, ICAM-1 and VCAM-1 are expressed on a variety of cell types including leukocytes, epithelial and endothelial cells. As cell surface glycoproteins of the immunoglobulin superfamily they act as mediators of firm adhesion between leukocytes and endothelium during immune responses. Accordingly, expression levels of ICAM-1 reach a maximum after 12 to 20 hours after stimulation and remain elevated for at least another 48 hours [Reinhart, 02].

Thus, the different expression kinetics of these adhesion molecules underline their diverse role in inflammatory processes observed in the endothelium.

NFκB and its inhibitor IκB.

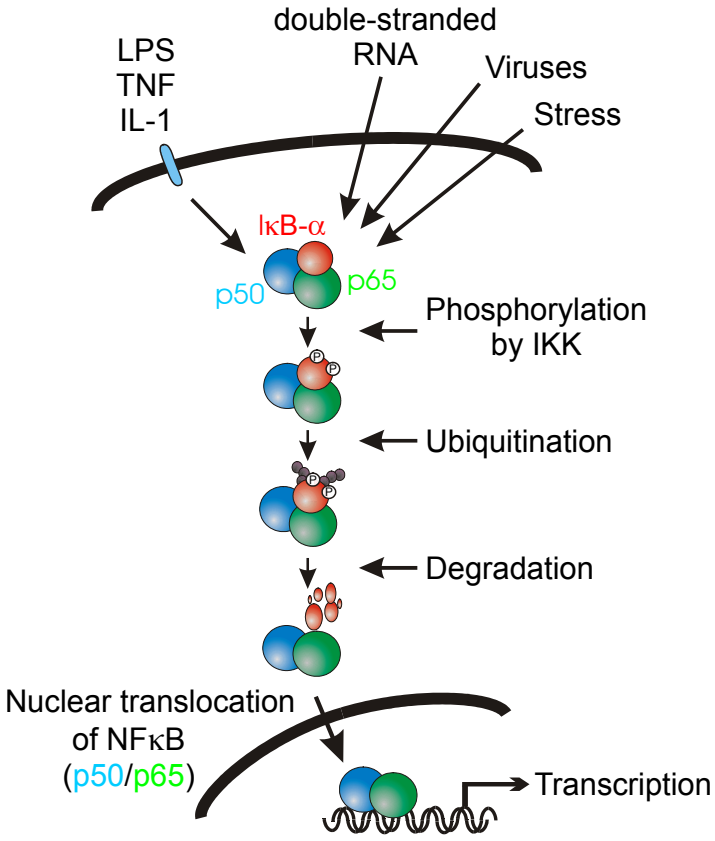

Figure 3. Mechanisms of NFκB activation.

NFκB is sequestered in the cytoplasm by the inhibitory IκB protein. Stimulation by a diverse array of pathogens and other inducers including viruses, lipopolysaccharide (LPS), tumor necrosis factor (TNF), interleukin-1 (IL-1) and stress-inducing agents leads to the activation of signalling cascades. They culminate with the activation of the IκB kinase (IKK) complex and phosphorylation of the inhibitor IκB. NFκB subunits which bind to DNA are released and translocate into the nucleus where they transactivate NFκB-responsive genes.

NFκB is a transcription factor expressed in numerous different cell types after stimulation or cell activation by a wide variety of stimuli like cytokines, crosslinking of surface molecules, viruses or viral proteins, to name only a few. A variety of different genes contains NFκB binding sites in their promoters. Cytokines and their receptors, cell adhesion molecules and many other proteins involved in various processes, including immune responses and inflammation, are important proteins encoded by NFκB-responsive genes.

NFκB activities are controlled by inhibitory proteins (Figure 3). Inactive NFκB is located in the cytoplasm of cells where it is complexed by the inhibitor of NFκB (IκB). Signal-dependent phosphorylation results in the ubiquitination of IκB protein and targets the cytoplasmic inhibitor to the ubiquitin-proteasome pathway where it is degraded [Chen, 95]. Dissociation of IκB from NFκB results in the translocation of the released transcription factor into the nucleus.

Mitogen-activated protein kinases.

Cells respond to extracellular signals by transmitting intracellular instructions and therewith coordinate an appropriate reaction. Usually, such reactions are accomplished through gene regulation, either downregulation or upregulation as in the increased expression of major HLA class II antigens leading to an amplified inflammatory response.

Alternatively, at the molecular level signal transduction is often achieved by activation or inhibition of regulatory proteins through phosphorylation by protein kinases. MAP kinases represent an important and rapid signalling cascade to transduce such extracellular signals into cellular responses (Figure 4).

In humans, three major groups of MAP kinases have been identified: the extracellular signal–regulated kinases (ERK or p42/44), the p38 MAP kinases, and the c-Jun amino-terminal kinases [Schaeffer, 99].

p38 is recognised as the MAP kinase that is activated in response to inflammatory stimuli. LPS, TNFα and IL-1 are exemplary stimuli inducing p38 activation in monocytes and endothelial cells eventually resulting in the proinflammatory phenotype of these cells. Namely endothelial E-selectin and COX-2, molecules associated with an inflammatory state, are expressed in a p38-dependent manner [Arbabi, 02].

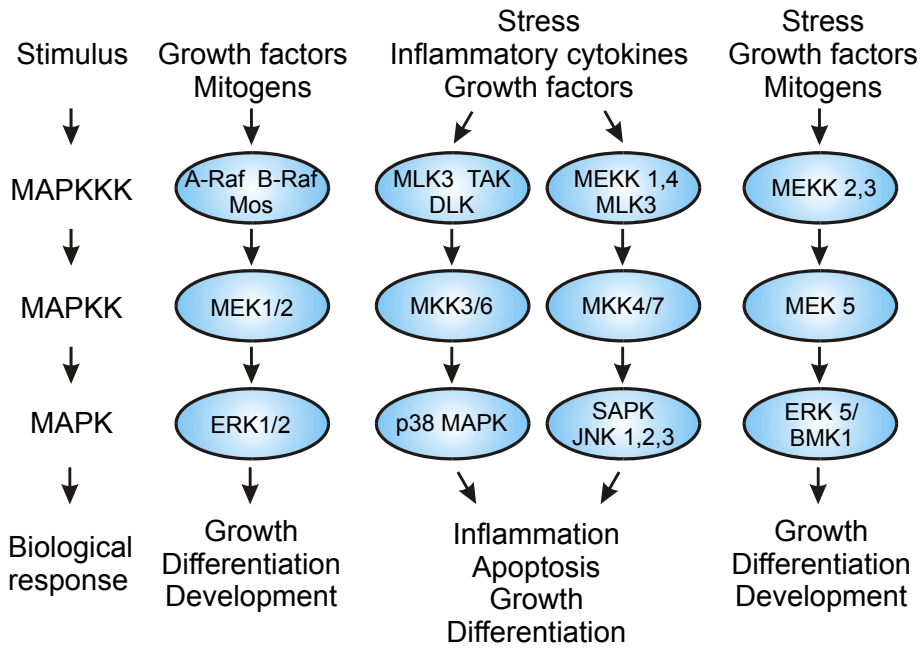

Figure 4. The mitogen-activated protein (MAP) kinase activation cascade.

The classic MAP kinase (MAPK) cascade consists of three intracellular protein kinase activation steps. Initiation involves the activation of MAP kinase kinase kinase (MAPKKK), including TGF-activated kinase (TAK), leucine zipper-bearing kinase (DLK), mixed lineage kinase (MLK) or MAP/ERK kinase kinase (MEKK). These Ser/Thr kinases phosphorylate and thereby activate MAP kinase kinases (MAPKK). Subsequently, MAPKK, such as MAP kinase kinase (MKK) and MAP/ERK kinase (MEK), activate MAP kinases by phosphorylation on two threonine and tyrosine residues. MAP kinases like extracellular signal-regulated kinase (ERK), stress-activated protein kinase (SAPK), c-Jun N-terminal (JNK) or big MAPK (BMK), as proline-directed protein kinases then activate other protein kinases and transcription factors such as the STAT family [Aaronson, 02; Arbabi, 02].

Thus, p38 is classically known as “stress-induced” MAP kinase, whereas p42/44, or ERK, is thought to be predominantly involved in the regulation of cell proliferation [Most, 03]. p42/44 is activated in response to LPS and various growth factors like TGF or PDGF. Moreover, adherence of monocytes and endothelial cells activates p42/44 through integrin-mediated phosphorylation [Clark, 98].

1.2.3 HUVEC as an infection model?

Still, there is no appropriate disease model to study either HCPS or HFRS. Even though hantaviruses cause lethal disease in newborn mice [Kim, 85], the disease does not mirror the disease observed in humans. Only recently, a small animal disease model for HCPS has been proposed [Hooper, 01]. However, the described lethal disease model in adult Syrian hamsters has been established for Andes virus only. HFRS-inducing hantaviruses, in contrast, do not cause disease in hamsters even when infected with high doses [Hooper, 01]. However, it has been demonstrated recently, that infection of cynomolgus macaques by wild-type PUUV

resulted in typical signs of HFRS including lethargy, anorexia, proteinuria and hematuria as well as cytokine responses, and virus-specific immune responses mimicked those seen in humans [Klingström, 02].

This lack of a suitable small animal model in addition to the general advantages that an *in vitro* model offers, such as the relatively simple and inexpensive realisation of a large number of experiments, justifies the use of such an infection model. Additionally, experiments can be specifically focused on a single cell type of interest.

Endothelial cells constitute not only the first barrier between the blood and the extravascular space. The endothelium also contributes significantly to a wide variety of cardiovascular functions including the regulation of permeability, blood pressure, transmigration of leukocytes and exchange of oxygen, carbon dioxide as well as metabolites. Endothelial integrity is required for proper function of the endothelium and mainly ensured by endothelial cell-cell junctions. Moreover, endothelial cells are target for and participate in immune reactions. For this reason, human endothelial cells are an obvious cell type to study hantavirus pathogenesis. Direct viral effects can be analysed and indirect effects such as soluble factors secreted by infected cells can be investigated.

While continuous cell lines exhibit characteristics that are similar to those of primary cultures, immortalised cells have aquired genetic changes that might alter cellular function. Therefore, primary cells, although less uniform and perpetual, provide a suitable means to study pathogenesis in a near-natural manner. HUVEC are such primary endothelial cells isolated from human umbilical cord veins [Jaffe, 73].

1.3 Immune mechanisms in hantavirus pathogenesis

The role of the immune response in the pathogenesis of human hantavirus infections is not entirely clear. Infection leads to the generation of antigen-specific activated T lymphocytes [Van Epps, 99; Van Epps, 02]. In both HCPS and HFRS, hantavirus antigen-specific cellular and humoral responses are present at the clinical onset of the disease [Peters, 99]. Besides observed changes in the cellular immune response such as increased concentrations of CD8-positive cells, increased levels of immune mediators such as TNFα or IFN-γ can be detected in patient's blood [Markotic, 99; Vapalahti, 01]. These observations, together with the obvious lack of any cytopathic disruption of the endothelium, support a role for immune-mediated mechanisms in the pathogenesis of hantavirus infections [Zaki, 95].

1.3.1 Intracellular responses

IFN pathways.

Once cells are successfully infected with hantaviruses their replication in cell culture is subsequently restricted by IFN induced by the viral infection itself [Pensiero, 92; Temonen, 95]. Still, hantaviruses are not particularly effective IFN inducers as shown by *in vitro* infections of human macrophages or serum levels of patient with HFRS [Krakauer, 94; Temonen, 95]. Externally added type I IFN, however, is likewise able to inhibit the replication in infected cells [Kanerva, 98; Nam, 03; Temonen, 95].

Many of the diverse biological effects of IFN are based on the binding of an IFN to its receptor. Subsequently, a rapid expression of genes that contain a conserved enhancer element, the so called Interferon Stimulated Response Element or ISRE, is induced. Genes containing an ISRE represent components of the antiviral defence such as OAS and the dsRNA activated PKR, cell surface proteins such as ICAM-1 or HLA class I and II molecules. The induced protein kinase, for instance, inactivates the eukaryotic initiation factor eIF-2 and thus inhibits the synthesis of new viral proteins. The expression of OAS leads to products which themselves activate endonucleases that in turn degrade viral RNAs [Williams, 91]. In fact, several cellular genes associated with an antiviral response such as IFN-induced OAS are upregulated after infection of human endothelial as well as human lung epithelial cells with hantaviruses [Geimonen, 02; Nam, 03].

Yet another IFN-regulated pathway involved in the cellular protection against viral infections includes so-called Mx proteins. These IFN-induced large GTPases interfere with the replication of a broad range of RNA viruses by perturbing the intracellular movement and functions of viral proteins [Frese, 96]. Recently, it has been shown in cell culture experiments that several members of the Bunyaviridae family are in fact inhibited by the Mx protein, including Puumala (PUUV), Hantaan (HTNV), and Sin Nombre virus (SNV) as well as Tula virus (TULV) [Kanerva, 96a]. Furthermore, it has been demonstrate that the human MxA protein intracellularly sequesters the viral N protein of La Crosse virus, another member of the *Bunyaviridae* thereby trapping an essential virus component in cytoplasmic inclusions. It is then unavailable for the generation of new virus particles [Kochs, 02]. For a complete inhibition of PUUV or TULV replication, however, high Mx expression

levels are required [Kanerva, 96a]. This could also be responsible for the relative inefficiency of IFN-α therapy in HTNV-infected humans [Gui, 87].

Other cytokines and chemokines.

In both HFRS and HCPS there is evidence of a strong cellular immune response, characterised by elevated levels of IFN-γ and TNFα in the blood of patients [Khaiboullina, 02; Peters, 99]. Apart from the induction of these cytokines *in vivo*, however, the selective induction of the chemokines RANTES (regulated on activation, normal T cells expressed and secreted) and interferon-inducible protein (IP)-10 in human microvascular endothelial cells after infection with HTNV may provide a clue to the role of the specific immune response in hantavirus pathogenesis [Sundstrom, 01]. Both RANTES and IP-10 are predominantely chemotactic for leukocytes, and IP-10 is essential for the development of a protective T_H1 immune response [Liu, 00]. Thereby, these chemokines provide a mechanism by which hantavirus infection enhance recruitment of activated monocytes and lymphocytes to the infected endothelium. These cells are indeed the major immune cell types found in lungs of HCPS patients as well as in kidney biopsies of HFRS patients [Cosgriff, 91; Zaki, 95].

Yet another protein involved in vascular permeability, VEGF, was recently shown to be upregulated after an infection with HTNV in human endothelial cells [Sundstrom, 01; Khaiboullina, 02]. As a potent chemoattractant for monocytes and as a highly specific enhancer of microvascular permeability, it could also play an important role in the immune-mediated endothelial dysfunction observed after hantavirus infection.

Apoptotic cell death.

Multicellular organisms can employ a number of defence strategies to combat viral replication, the most dramatic being clearance of infected cells by apoptotic processes. The overall cost to the viability of an organism of losing infected cells by apoptosis may be small if the dying cells can be substituted.

Apoptosis, or programmed cell death, is a regulated physiological process leading to cell death characterised by cell shrinkage, membrane blebbing and DNA fragmentation. This active process involves the coordinated activity of a variety of proteins, separated into activators, effectors, and negative regulators, which are

brought together in different signalling pathways. In fact, cellular survival requires active inhibition of apoptosis.

Akt, referred to as protein kinase B (PKB) or Rac, plays a critical role in controlling the balance between survival and apoptosis. This protein kinase is phosphorylated and activated by various growth and survival factors including cytokines like IL-6, which is produced in response to viral infections, or VEGF [Mogensen, 01]. Akt then functions to promote cell survival by inhibiting apoptosis by phosphorylating and thereby inactivating several targets such as caspase-9. In fact, it has been shown recently, that Akt signalling promotes endothelial cell survival by inhibiting MAP kinase p38-dependent apoptosis. Thus, cross-talk between Akt and p38 pathways may regulate the balance between cell survival and apoptosis and is a new mechanism to explain the survival actions of Akt [Gratton, 01].

Alternatively, Epstein-Barr virus (EBV) or human herpes virus-8 (HHV-8) have been shown to express bcl-2 homologues and thereby promote cell survival by blocking apoptosis [Korsmeyer, 99]. The antiapoptotic bcl-2 has the ability to exert a survival function in response to a wide range of apoptotic stimuli by blocking mitochondrial release of cytochrome c [Yang, 97; Kluck, 97].

1.3.2 Humoral and cellular immune response

Humoral immune responses to hantavirus antigens have been studied extensively. During the early phase of the disease an IgM response against all structural proteins of the virus can be detected in the blood of HFRS patients. The N protein, however, is presumed to be the major target of this early antibody response. Levels of IgM antibodies then decline while levels of virus-specific IgG antibodies increase simultaneously [Groen, 92; Lundkvist, 93]. Antibody titers against the two envelope glycoproteins, G1 and G2, then dominate over the N-specific antibody response [Kanerva, 98]. Thus, IgG antibodies are mainly induced by the glycoproteins G1 and G2, and it could be concluded that these proteins are major inducers of the humoral immune response [Khaiboullina, 02].

Pathological manifestations seen during HFRS has been suggested to be the result of virus-specific CTL responses [Van Epps, 02]. Increased numbers of stimulated CD8-positive T lymphocytes have been observed in the blood from patients with acute severe hantavirus-associated disease [Nolte, 95]. Surprisingly, high frequencies of memory CD8-positive T lymphocytes can be found a long time after the clinical hantavirus infection has resolved [Van Epps, 02]. Moreover, CD8-

positive T lymphocytes have been detected in association with hantavirus-infected lung endothelial cells by immunohistochemical analysis of specimens derived from HCPS patients [Nolte, 95; Zaki, 95]. In kidney biopsies of patients with HFRS infiltrating CD8-positive T lymphocytes have been localised near the tubuli [Temonen, 96]. Finally, hantavirus-infected endothelial cells have been shown to secrete chemokines that attract T lymphocytes [Sundstrom, 01].

Activation of antiviral T lymphocytes, particularly in a primary response, depends critically on the proper function of dendritic cells. A growing number of viruses such as human cytomegalovirus (CMV) or measles virus are known to infect human dendritic cells and interfere with the function by a variety of mechanisms [Fugier-Vivier, 97; Raftery, 01]. Interestingly, immunohistochemistry performed on samples derived from HCPS patients demonstrated that hantavirus antigen colocalises with follicular dendritic cells in the spleen and in lymph nodes [Zaki, 95]. This finding hinted at hantavirus replication in dendritic cells. A subsequent release of proinflammatory cytokines such as TNFα and IFN-α could then enhance the observed hantavirus-induced endothelial cell leakage. It is furthermore believed that natural killer (NK) cells represent an important part of the innate immune response against viral infection [Guidotti, 01]. The observed decrease in the number of NK cells in the blood of HFRS patients [Lewis, 91; Linderholm, 93] might be due to the migration of these cells into infected tissue. Specific cytokine signals produced by infected cells then trigger the maturation of naive NK cells *in situ* into mature NK cells [Matsukawa, 00].

Again, these findings suggest that cellular immune mechanisms may play a more significant role than direct effects in the pathogenesis of endothelial dysfunction induced by hantavirus.

1.3.3 Viral immune evasion

Viruses have evolved elegant mechanisms to evade detection and destruction by the host's immune system. Strategies of viral immune evasion are directed towards the cellular and humoral immune response as well as innate immune effector functions. Finally, some viruses have the additional capacity to hide from the host's immune defence by establishing latency.

Appropriate mechanisms comprise restriction of HLA class I molecules which blocks the presentation of viral antigen thereby interfering with the cellular immune response. The HLA antigen-presentation pathway itself can also be blocked by viral

gene products at multiple levels. The spectrum of employed strategies ranges from the retention of HLA class I molecules in subcellular compartments to interference with proteasomal degradation to the prevention of peptide transport by the transporter associated with antigen presentation (TAP) [Alcami, 00]. However, disrupting the functionality of dendritic cells is yet another mechanism to impair the host's immune defence [Bhardwaj, 97].

On the other hand, downregulation of HLA class I molecules on the cell surface results in elimination of the infected cell by natural killer cells [Ljunggren, 90]. Therefore, some viruses avoid natural killer cell-mediated killing by expression of HLA homologues that function as viral dummies to prevent the expected cytotoxicity [Farrell, 97]. Yet another strategy targets the IFN system, and in fact, this appears to be a major strategy employed by different viruses to subvert the antiviral response [Levy, 01; Vossen, 02]. Viruses have evolved mechanisms to inhibit IFN signalling by disarming the host immune defence. In fact, many virus genomes contain "anti-immune genes" that control the synthesis of host cell proteins, including cytokine homologs and cytokine receptor homologs, for example.

The comparatively small coding capacites of hantaviruses, however, limit the number and extent of potential immune evading mechanisms. In fact, little is known about the precise interplay between hantaviruses and the host defence system. The implication of this complex interplay on viral pathogenicity, viral clearance, viral immunity and persistence is yet to be clarified.

1.4 Objectives

Hemorrhagic fever viruses modify hemostatic mechanisms in two separate ways. The first is through direct action on cells involved in vascular hemostasis, and the second is through immunological and inflammatory pathways. However, the mechanisms underlying virus-associated pathogenicity are largely unknown.

Therefore, the following questions concerning the immunopathogenesis of hantavirus infections should be addressed in this study:

1. Are hantaviruses able to directly activate human endothelial cells?

The capacity of pathogenic and rather nonpathogenic hantaviruses to induce an antiviral response in endothelial cells should be compared. For this purpose, the growth of HTNV and TULV should be comparatively analysed in HUVEC and Vero E6 cells. Expression of antigen presentation molecules and the production of type I IFN in HTNV- and TULV-infected cells should be investigated. Moreover, induction of the IFN-induced MxA protein by HTNV or TULV should be explored in HUVEC.

Furthermore, the analysis of virus-directed cellular responses such as general induction of inflammatory pathways or specific induction of adhesion molecues should provide insight into disease mechanisms. These data should facilitate an essential understanding of virus-cell interactions that are likely to contribute to disease.

2. Are human dendritic cells susceptible to hantavirus infection? How are they affected by an infection with hantaviruses?

As dendritic cells and macrophages play a major role in the induction and regulation of the innate and adaptive immune response, the tropism of these viruses for the antigen-presenting cells (APC) should be studied. The susceptibility to an infection as well as the ability to support a productive infection should be determined.

Prior to the infection experiments appropriate safety precautions ought to be established. Therefore, an additional objective of the present study should be the verification of inactivation and depletion procedures already in use for fixation, inactivation and removal of infected cells or viruses. These procedures should be assessed concerning subsequent utilisability of treated materials in research assays outside biosafety level 3 containment laboratory.

2 Materials and Methods

2.1 Cell culture

2.1.1 HUVEC

Human umbilical vein endothelial cells (HUVEC) were prepared by a method described previously [Jaffe, 73] and modified recently [Thornton, 83].

Cultures of HUVEC were grown on gelatin-coated plates. Confluent cells in the second passage were used for experiments. HUVEC were maintained in MCDB131 (Gibco BRL, Karlsruhe, Germany) supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml amphotericin B, 4.5 mM L-glutamine and 20 μg/ml endothelial cell growth factor (ECGF).

2.1.2 Vero E6

Vero E6 (Vero C 1008, CRL 1586, ATCC) cells, african green monkey kidney cells, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 IU penicillin, 100 μg/ml streptomycin and 4.5 mM L-glutamine. Medium and FCS were certified endotoxin-free by the manufacturer's.

2.1.3 Peripheral blood mononuclear cells and dendritic cells

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Germany) using buffy coats that were supplied by the German Red Cross, Berlin. In a further step, monocytes were separated from PBMC by adhesion to plastic for 1 h at 37°C, with contaminating cells being removed by four washing steps. More than 75% of the resultant cells expressed the CD14 markers. Immature dendritic cells were generated from adhesion-purified monocytes by growth for 6 days in RPMI 1640 with with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 4.5 mM L-glutamine and supplemented with 800 U/ml of both granulocyte-macrophage-colony-stimulating factor (GM-CSF) and interleukin (IL) -4. Where required, mature dendritic cells were generated from these cells by incubation with 1,000 U/ml tumor necrosis factor α (TNFα) in addition to GM-CSF and IL-4 for another two days.

2.2 Working with the virus

Hantaviruses present a hazard to all laboratory workers engaged in their study, and appropriate precautions have to be taken in order to prevent infection. These include wearing protective clothing including, at the minimum, mask, closed footwear, and gloves. The handling of the virus is carried out under a hood so designed that the air flows away from the worker to the exhaust, and on the way passes through a filter system. All cultures, stocks, and other regulated wastes have to be decontaminated before disposal by an approved decontamination method, such as autoclaving.

2.2.1 Virus growth and stock production

Stocks of HTNV, strain 76-118 [Lee, 78], and TULV, strain Moravia [Plyusnin, 95], were propagated on Vero E6 cells. Cells grown in a 75 cm^2 cell culture flask (Nunc, Wiesbaden, Germany) were inoculated with 0.4 ml of a preexisting virus stock in a total volume of 15 ml cell culture medium and incubated for 12 days in a humidified atmosphere at 37°C. The cell culture supernatant was harvested, another 5% FCS added (final concentration of 10%), aliquoted and stored at –80°C until further use. Concentrated viral stocks were prepared by pelleting virus from supernatant of infected cells at 130,000 g for 4 hours at 4°C. Virus pellets were resuspended in Tris-HCl buffer pH 7.6 supplemented with 5% FCS and stored at -80°C until further use. Virus stocks were free of mycoplasma contamination as tested by PCR.

2.2.2 Titration of virus stock

For virus titration a focus assay was performed essentially as described by Heider et al., 2001. Briefly, Vero E6 cells were seeded into six-well plates. When confluent, cells were inoculated with 0.2 ml/well HTNV diluted in dilution medium containing Hank's balanced salt solution (HBSS, GibcoBRL, Karlsruhe, Germany) supplemented with 2% HEPES (GibcoBRL), 2% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (GibcoBRL). After virus adsorption for one hour at 37°C wells were overlaid with 2.5 ml/well of a pre-warmed (42°C) 1:1 mixture of 1% LE agarose (Cambrex, East Rutherford, NJ) and basal Eagle's medium supplemented with 2.5% HEPES, 10% FCS and antibiotics. Plates were incubated for 7 days in a humidified atmosphere at 37°C. The overlay was then discarded, cells washed twice with PBS

supplemented with 0.15% Tween-20 and finally fixed for 8 minutes with methanol. After the methanol has been removed cells were allowed to dry and again washed two times. To detect hantaviral antigens in HTNV-infected cells an anti-DOBV-positive, HTNV-cross-reactive human serum, kindly provided by Morten Schütt, Lübeck, and horseradish-peroxidase (HRP)-labelled anti-human immune globulin (Dianova, Hamburg, Germany) were used. TULV antigens were determined by incubating cells with a rabbit serum raised against *E. coli*-expressed N protein of TULV, strain Malacky [Sibold, 99]. The immune reaction was detected by addition of a chemiluminescence substrate (Super Signal Ultra, Pierce, IL) and documented using a DIANA II-CCD camera with an integrated, software controlled filter system (Raytest Isotopenmessgeräte, Straubenhardt, Germany).

2.2.3 Infection of cells

For infection, equal quantities of viable virus or UV-inactivated HTNV (mock-infection) were allowed to adsorb to HUVEC for 1 hr at 37°C. UV-irradiation for 5 minutes completely inactivated HTNV corresponding to a reduction of the viral titer of at least 6 log scales (data not shown). Subsequently, a proof of infection was performed using a focus assay in which virions from the supernatant of infected cells were identified as being infectious (see 2.2.2). Alternatively, viral antigen was detected in lysates of infected cell using Western blot technique (see 2.4) or in infected cells by indirect immunofluorescence assay (see 2.3.2).

2.2.4 Safety precautions

Preparation of HTNV-containing samples during chemical inactivation procedures.

To evaluate the inactivation capacities of the fixation agents (methanol, paraformaldehyde, acetone/methanol) used to treat HTNV-containing samples, first washing solutions and cell lysates obtained after the fixation procedure (Figure 5) were collected. These samples were analysed for remaining replication-competent HTNV in a subsequent “infectivity” focus assay. Moreover, the use of detergent-containing cell lysis buffer was tested for its efficiency to reduce viral infectivity in a similar way.

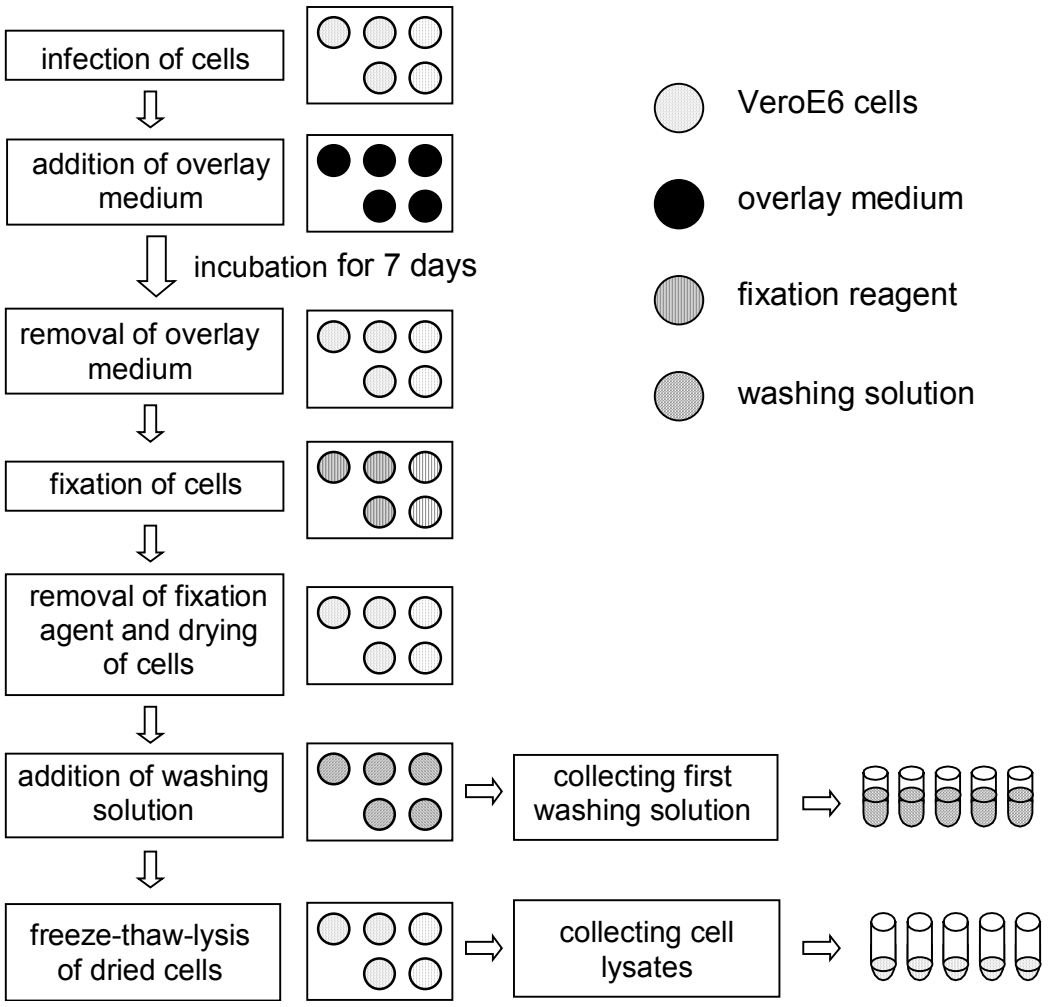

Figure 5. Viral inactivation and depletion procedures.

Flowcharts of virus infection, subsequent fixation of infected cells and preparation of samples from infected cells after the fixation procedure.

For the fixation procedure (Figure 5) cells were grown in six-well plates and then infected with serial dilutions of HTNV (usually 10, 100, 1,000, and 10,000 fold in dilution medium, otherwise as indicated; wells 1-4). As a negative control 200 µl of dilution medium alone was added to the 5th well. The 6th well was left empty, leaving space for a following positive control well on the plate of the subsequent “infectivity” focus test.

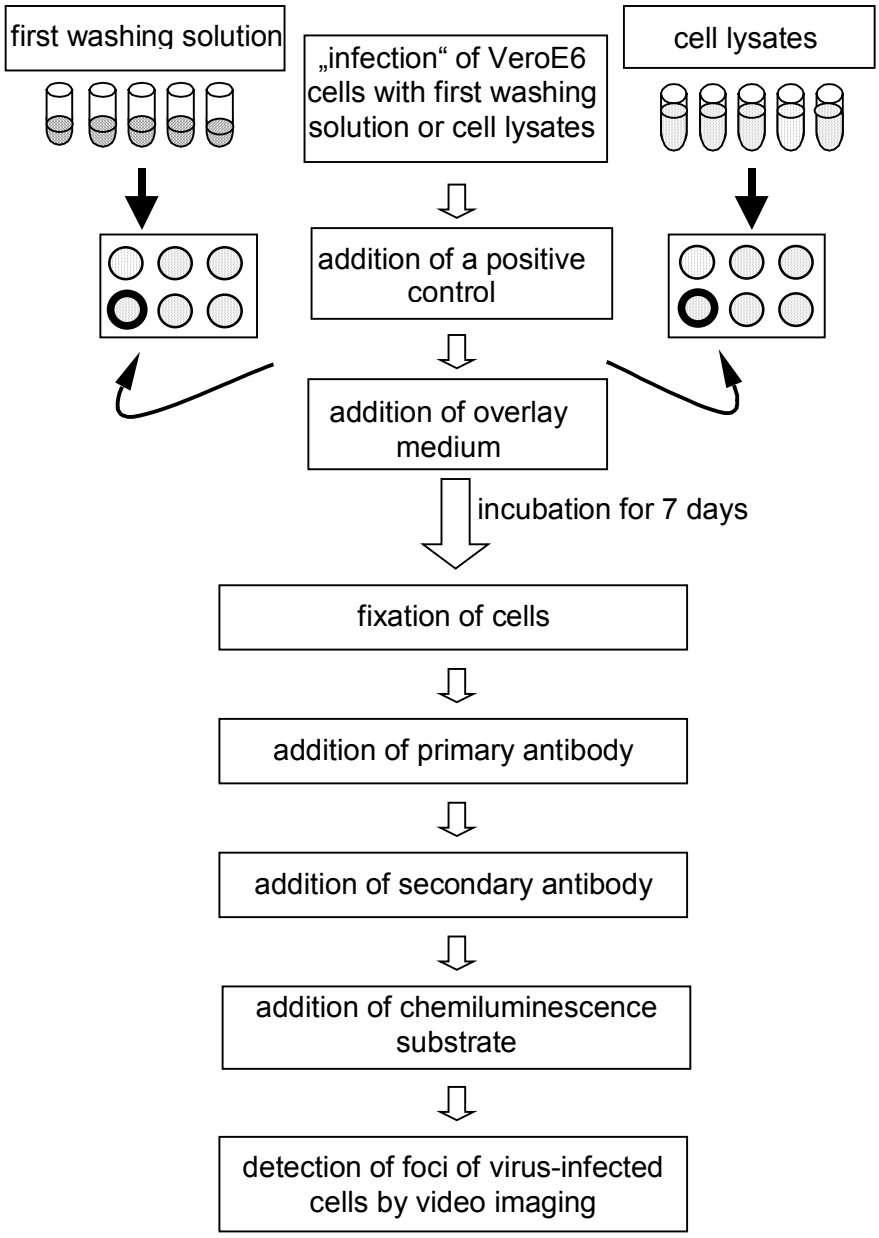

Figure 6. "Infectivity" focus assay.

Flowchart of the subsequent “infectivity” focus assay for detection of infectious particles in lysates and first washing solutions of infected cells after fixation.

After seven days of incubation at 37°C and 5% CO_2, the agarose overlay was discarded by gently injecting 1 to 2 ml of PBS under the agarose layer, cells were washed once with PBS and treated with the respective agent. Generally, cells were incubated with 2 ml/well of absolute methanol for 8 minutes, 1% paraformaldehyde for 20 minutes, or acetone/methanol (1:1) for 10 minutes.

Cells were then allowed to dry and, finally, 200 µl/well of PBS (with 5% FCS) were added and eventually collected as first washing solution. In case of untreated samples cells were washed with PBS only (first washing solution). To avoid the loss of any infectious HTNV and thereby retaining maximal viral titers in this control titration, drying of cells was left out.

After treatment, cell lysates were obtained by freeze-thaw-lysis in order to avoid loss of viral infectivity by denaturing agents and after addition of 5% FCS lysates were eventually stored at –80°C (Figure 5). Alternatively, lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.25 mM EDTA, 1 mM PMSF and 1% NP40™ was used to extract proteins from virus-infected cells. These lysates were ultracentrifugated at 130,000 × g and 4°C for 2 h. Pellets were resuspended in 500 µl 50 mM Tris-HCl (pH 7.6) with 5% FCS and stored at –80°C until further use.

Replication-competent HTNV was detected in the subsequent “infectivity” focus assay (Figure 6). An internal positive HTNV control of defined titer was added on each six-well-plate.

Physical procedures of virus inactivation and depletion.

For virus depletion by filtration centrifugal filter devices containing low protein-binding Omega™ membranes with 300 kDa or 1,000 kDa cut-off were used according to the manufacturer’s instruction (Pall Life Sciences, Dreieich, Germany). Filtrates were supplemented with 5% FCS and stored at –80°C until further use in the “infectivity” focus assay.

Alternatively, virus stock solution (0.5 ml) was transferred into a small petri dish and placed directly on the work space of a UV Transilluminator equipped with 8-watt tubes (Vilber Lourmat, Torcy, France). Inactivation of hantavirus stock was performed by UV-irradiation for 1, 3 and 5 minutes at 312 nm.

2.3 Immunological methods

2.3.1 Flow cytometry

For surface immunofluorescence analysis by flow cytometry cells in suspension were washed once with ice-cold washing solution (PBS with 1% heat-inactivated fetal calf serum and 0.05% sodium azide) before being resuspended with the first antibody in ice-cold blocking solution (PBS with 10% heat inactivated fetal calf serum and 0.2% sodium azide) for 1 hour. Cells were then washed twice in ice-cold washing

solution and the staining procedure was repeated with fluorescein isothiocyanate (FITC)-coupled goat anti-mouse secondary antibody. After the final staining step cells were washed twice in ice-cold washing solution and resuspended in 2 ml PBS with 1% formaldehyde. Tubes were left at 4°C overnight before being centrifugated. The cells were resuspended in 200 µl of PBS with 0.2% formaldehyde before being measured.

Antibodies. The following antibodies were used for flow cytometry: anti-ICAM-1 (clone HA58) and anti-HLA-DR, DP, DQ (clone Tü39) were purchased from PharMingen, Heidelberg, Germany, anti-HLA I (clone W6/32) was obtained from Serotec, Oxford, UK, and anti-VCAM-1 (clone 1G11) was purchased from Immunotech, Marseille, France. As a non-reactive antibody, i.e. a non-specific binding control or isotype control, anti-CD1a (clone NA1/34) from Biozol, Eching, Germany, was added. As secondary antibodies FITC-coupled goat anti-mouse and FITC-coupled swine anti-rabbit serum (DAKO, Hamburg, Germany) were employed.

Analysis. A FACScalibur®, a Fluorescence Activated Cell Sorter and analyser, from Becton Dickinson, Heidelberg, was used to quantify expression levels of labelled molecules on the surface of the cells. In general, expression levels of a certain molecule were presented as mean fluorescence intensity (MFI) against the overall number of cells. The provided Cell Quest® software allowed analysis of the obtained data.

2.3.2 Immunofluorescence assay

Method. Since F-actin is concentrated along the margains of endothelial cells rhodamine-labelled-phalloidin was used to visualise interendothelial gap formation as well as changes in cell morphology. To this end, cells were fixed in paraformaldehyde and then permeabilized using 0.1% Triton X-100 for 5 min followed by three washing steps with phosphate-buffered saline (PBS). Eventually, F-actin was directly stained with phalloidin.

To prove a recent infection by immunocytochemistry hantavirus-infected cells were transferred onto slides, incubated overnight at 37°C and then fixed with acetone/methanol (1:1) at 4°C for 30 min. Slides were washed thrice in PBS and incubated for 1 h at room temperature with the first antibody in blocking solution

(PBS with 10% heat-inactivated species-specific serum). Cells were then washed again thrice in PBS and subsequently stained with the secondary antibodies in blocking solution. Finally, cells were washed, embedded in mounting medium and slides were stored at 4°C before being analysed.

Accordingly, cell surface molecules were stained on infected cells, however, without prior permeabilisation.

Antibodies. The following antibodies were used for immunocytochemistry: anti-ICAM-1 (clone HA58) was purchased from PharMingen, Heidelberg, Germany, and anti-NFκB (clone C-20) was obtained from Santa Cruz Biotechnology, California. For detection of HTNV the monoclonal antibody 1C12 specific for the hantaviral N protein [Lundkvist, 91; Lundkvist, 02] or a HTNV-crossreactive human serum derived from a hantavirus (Dobrava)-infected patient was used. Expression of TULV antigens was determined by incubating cells with a rabbit serum raised against *E. coli*-expressed N protein of TULV, strain Malacky [Sibold, 99]. As secondary antibodies FITC-coupled goat anti-mouse and FITC-coupled swine anti-rabbit serum (DAKO, Hamburg, Germany) were employed. F-actin was stained with rhodamine-labelled-phalloidin Alexa 488 (Molecular Probes, Eugene, OR).

Analysis. Slides were sealed and examined in an IMT-2 fluorescence microscope (Olympus Optical, equipped with an Olympus OM-4 camera) with an Olympus 63× objective.

2.3.3 ELISA

Quantification of type I IFN. Concentrations of IFN-α and IFN-β in supernatants harvested from infected cells were quantified using a sandwich ELISA kit (R&D systems, Wiesbaden, Germany) according to the manufacturer's instructions. Briefly, a microplate precoated with polyclonal antibodies specific for IFN-α or IFN-β, respectively, was used. Standards and samples were pipetted into the wells and any IFN present was bound by the respective immobilised antibodies. After any unbound substances were washed away, an enzyme-linked polyclonal anti-IFN-α or anti-IFN-β antibody was added to the wells. Unbound antibody-enzyme reagent was washed away and a substrate solution was added to the wells. Absorbance was determined

at 450 nm and intensity increased in proportion to the amount of IFN bound in the initial step.

Quantification of cell surface adhesion molecules. Expression of adhesion molecules on monolayers of human endothelial cells was determined with a cell-surface ELISA technique [Krüll, 96]. Briefly, confluent HUVEC monolayers in 96-well flat-bottom microtiter plates were washed and infected with HTNV or mock-infected. Finally, cells were fixed with 4% paraformaldehyde. Human Ig was used to reduce nonspecific binding, and primary antibodies were added for 30 min. Thereafter, the cells were washed three times and exposed to a horseradish peroxidase-conjugated rabbit anti-mouse Ig antibody for 30 min. Finally, cells were washed again, o-phenylenediamine was added for 5 min and absorbance was determined at 492 nm.

Antibodies. The following antibodies were used for cell surface ELISA technique: anti-ICAM-1 (clone HA58) was purchased from PharMingen, Heidelberg, Germany, and E-selectin (clone H18/7) was obtained from Serva, Heidelberg, Germany.

2.3.4 TUNEL assay

Apoptosis of endothelial cells was visualised by immunohistochemical detection of DNA strand breaks [Gavrieli, 92]. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique was used to detect endothelial cell apoptosis (Boehringer, Mannheim, Germany). Briefly, hantavirus-infected or mock-infected endothelial cells cultured on chamber slides were fixed in freshly prepared paraformaldehyde (4% in PBS, pH 7.4). After blocking of endogenous peroxidase, cells were permeabilized, TUNEL reaction mixture was added, and peroxidase substrate was used to visualise apoptotic nuclei. Slides mounted on glass coverslips were analysed under light microscopy (Olympus Optical, equipped with an Olympus OM-4 camera) and documented on Kodak TMY 400 film.

2.3.5 IFN bioassay

For quantification of IFN-activity a bioassay was conducted [Yousefi, 85]. For this purpose supernatant from infected HUVEC were transferred to A549 indicator cells (3 x 10^4/well). On the next day, cells were infected with encephalomyocarditis virus (EMCV). After 26 to 30 h medium was removed. To quantify the cytopathic effect, cells were fixed with 4% glutaraldehyde and stained with 1% crystal violet. The

dye was solubilised in 33% acetic acid, and the optical density of the eluate was measured at 570 nm in a Labsystem Multiscan MS ELISA reader. The amount of IFN is given as international reference units per milliliter, using exogenously added National Institutes of Health human IFN as a reference. Polyinosinic-polycytidylic acid (poly I:C) obtained from Sigma, München, Germany, was used to stimulate production of type I IFN in control cells.

2.4 Proteinchemistry

2.4.1 Lysates

Cell extracts were prepared by lysing cells in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, 1% Triton X-100 (wt/vol), and protease inhibitors (2 µg/ml leupeptin, µg/ml antipain, and 2 µg/ml pepstatin). Cell lysates were briefly centrifuged to remove cell debris. Total protein concentrations were determined according to the Bradford procedure [Bradford, 76], using the reagent kit and manufacturer's protocol purchased from BioRad Laboratories, Richmond, CA. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilise the anionic form of the dye, causing a visible color change. Samples were then resuspended in gel-loading buffer according to Laemmli and boiled for 5 min.

2.4.2 SDS-PAGE

Proteins in the mass range from 30 to 150 kDa were separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) with 50 µg total protein per lane and run at 120 V (Mini-Protean III, Bio Rad, München). All gels were run with Rainbow molecular weight marker RPN 800 purchased from Amersham Life Science, UK.

2.4.3 Western blot

Method. Following electrophoretic separation, proteins were electrotransferred onto Immobilon™-P transfer membranes (Millipore, Schwalbach, Germany) in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Thereafter, blots were blocked for 1 h at room temperature in high-salt Tris-buffered saline, consisting of 50 mM

Tris-HCl (pH 8.0), 500 mM NaCl, 0.05% Tween 20 (wt/vol), and 5% skim milk (wt/vol). Blots were incubated overnight at 4°C with specific primary antibodies. Blots were then washed thrice with high-salt Tris-buffered saline, followed by incubation with appropriate secondary antibodies for 1 h at room temperature. After final washing steps, detection was performed by enhanced chemiluminescence (ECL; Amersham Life Science, UK) or by visualisation of fluorescence-labelled secondary antibodies.

Antibodies. The following antibodies were used for Western blot analysis: anti-β-actin antibody (ab 6276) was purchased from Abcam ltd., UK and anti-MxA monoclonal antibody M143 was kindly provided by Otto Haller, Freiburg, Germany [Flohr, 99].

anti-IκB-α (clone C-21) and AKT (Ser 473) were obtained from Santa Cruz Biotechnology, California, and anti-bcl-2 (clone 7) was purchased from BD Biosciences, Heidelberg. Antibody 1C12 specific for hantavirus N protein was kindly provided by Åke Lundkvist, Stockholm, Sweden.

For the proof of infection HTNV-crossreactive human serum derived from a hantavirus (Dobrava)-infected patient was used. Expression of TULV antigens was determined by incubating cells with a rabbit serum raised against *E. coli*-expressed N protein of TULV, strain Malacky [Sibold, 99].

MAP kinases p38 and p42/44 as well as phospho-specific p42/44 (Tyr 204) and phospho-specific p38 (Tyr 182) were purchased from New England Biolabs, Beverly, MA. STAT-1 and phospho-specific STAT-1 (Tyr 701) were obtained from Biosource, California.

Peroxidase-conjugated rabbit anti-mouse IgG (Amersham Pharmacia Biotech, Dreieich, Germany) and IRDye 800- or Cy5.5-labelled secondary antibodies (Odyssey infrared imaging system; LI-COR, Lincoln, NE) were used as appropriate.

2.5 Molecular biology

2.5.1 Real-time PCR

To quantify levels of mRNA of molecules of interest in hantavirus-infected or mock-infected cells real-time PCR was conducted. This was kindly performed by Thomas Giese in Heidelberg.

RNA sample preparation. Cells (10^6) were lysed with 300 µl of MagnaPure lysis buffer (Roche Applied Science, Mannheim, Germany) and samples were frozen at –80°C. After thawing, lysates were mixed and transferred into MagnaPure sample cartridges and mRNA was isolated with a MagnaPure-LC device using a standard protocol. The elution volume was set to 50 µl. One aliquot of 8.2 µl RNA was reversely transcribed using AMV-RT and oligo-(dT) as primer (first strand cDNA synthesis kit for RT-PCR, Roche Applied Science) according to the manufacturer's protocol in a thermocycler.

After termination of cDNA synthesis, the reaction mix was diluted to a final volume of 500 µl and stored at –20°C until PCR analysis.

LightCycler PCR. Target sequences were amplified using LightCycler Primer Sets (Search-LC, Heidelberg, Germany) with the LightCycler FastStart DNA Sybr Green I Kit (Roche Diagnostics) according to the manufacturer's protocol. RNA input was normalised by the average expression of the housekeeping genes encoding β-actin and cyclophilin B. Copy numbers were calculated from a virtual standard curve, obtained by plotting a known input concentration of a plasmid to the PCR-cycle number (CP) at which the detected fluorescence intensity reached a fixed value. The data of two independent analyses for each sample and parameter were averaged and presented as adjusted transcripts/µl cDNA or as ratios to control values.

2.6 Statistical methods and formula

The paired students t-test was used to analyse inter-group differences. A value of $P<0.05$ was considered significant. The % inhibition of HLA class I-enhancement on HUVEC by anti-IFN-β antibodies in Figure 32 was calculated on the basis of the respective mean fluorescence intensity (MFI): % inhibition = (HTNV-uninfected)-(HTNVanti-IFN-β-uninfected)/ (HTNV-uninfected) x100.

3 Results

3.1 Safety aspects.

The major route of transmission of wildtype-hantaviruses to humans is via aerosolised excreta from persistently infected rodent reservoir hosts. Therefore, certain groups of people are at high risk for hantavirus infections due to their residence and profession like farmers, rodent trappers, mammalogists and soldiers in the field. Our infection studies clearly demonstrated the capacity of cell-culture adapted hantaviruses to readily infect and replicate in human endothelial cells. Therefore, experimental work on hantaviruses including infected tissues and viral cell culture requires safety level 3 containment laboratories [CDC, 94].

In order to be able to use samples for subsequent cell biological, virological and immunological investigations outside the safety level 3 containment laboratory inactivation or depletion procedures for hantaviruses had to be established.

3.1.1 Hantaan virus is susceptible to a broad range of chemical inactivation procedures.

Chemicals classically used for fixation of mammalian cells allowing subsequent use of samples for different applications like IFA, cell surface ELISA, flow cytometry analysis, immunoblot or focus assay were tested for their ability to inactivate HTNV. To estimate the required incubation time and dosage of these agents needed to completely inactivate HTNV the kinetics of inactivation were studied. The experimental design for preparation of first washing solutions and cell lysates as well as the subsequent analysing procedure are given in Figure 5 and 6.

Methanol fixation.

Methanol fixation for 8 minutes is classically used to fix cells for further use in hantavirus focus test or focus reduction neutralisation assay, FRNT [Heider, 01].

The first washing solution after fixation and cell lysates obtained by freeze-thaw-lysis were analysed separately. Fixation of HTNV-infected cells with methanol for 8 minutes depleted the number of focus forming units (FFU) in cell lysates completely, i.e. by at least 7 log scales (Figure 7 b and Table 1). In line with this, all washing solutions obtained from HTNV-infected and subsequently methanol-fixed cells did not

show any remaining infectious HTNV (Figure 7 e and Table 1). These data were confirmed both for washing solution and cell lysate in three independent experiments.

Paraformaldehyde fixation.

To prevent destruction of the cell surface for the analysis of outer membrane proteins in a cell surface ELISA cells were treated with 1% paraformaldehyde for 20 minutes at room temperature. Cell lysates and first washing solutions were tested in three and two independent experiments, respectively, for remaining infectious virus.

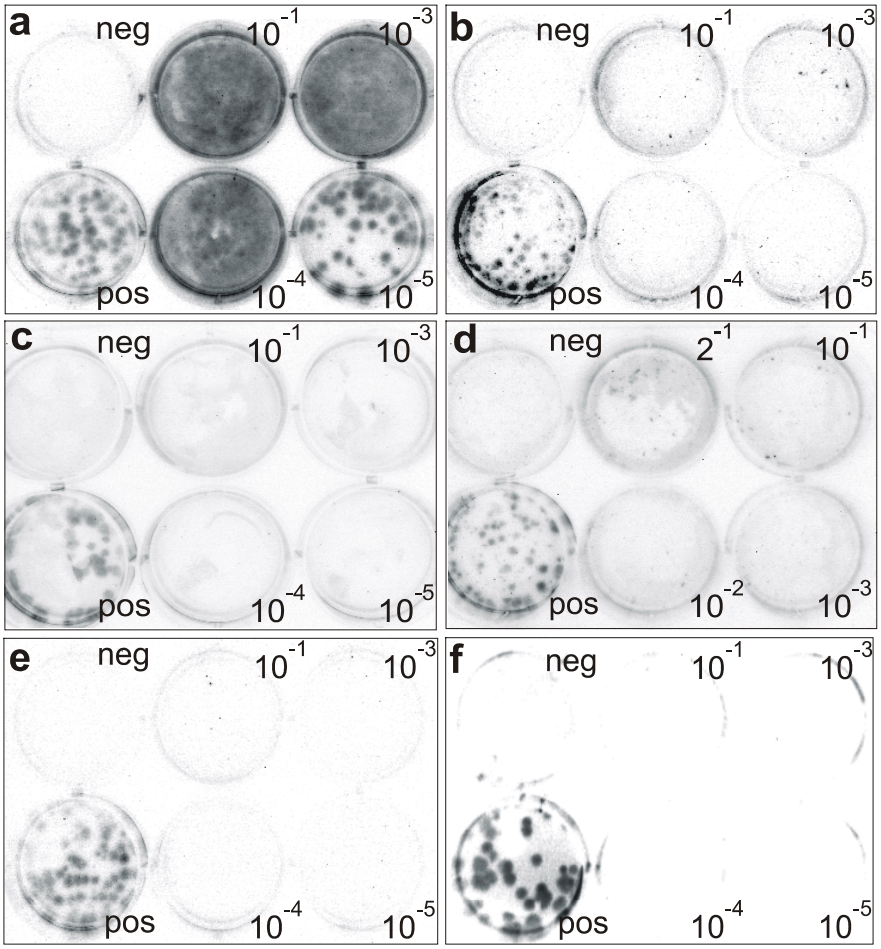

Figure 7. Inactivation of HTNV infectivity in lysates of infected cells and first washing solutions after fixation.

Vero E6 cells were inoculated with HTNV (dilutions as indicated) or left uninfected (upper left well; neg). Cell lysates obtained 7 days later without prior fixation (a) or after fixation with methanol for 8 minutes (b), paraformaldehyde for 20 minutes (c), and acetone/methanol for 10 minutes (d) were tested for remaining infectivity in a focus assay on fresh Vero E6 cells. Simultaneously, the first washing solutions after fixation of cells with methanol for 8 minutes (e) and paraformaldehyde for 20 minutes (f) were collected and screened for remaining infectivity in a focus assay on fresh Vero E6 cells. In all experiments an internal positive control with 200 FFU/well (lower left well; pos) was added.

Lysates of HTNV-infected cells treated for 20 minutes with 1% paraformaldehyde did not contain any remaining infectious virus (Figure 7 c). Similarly, infectious virus could not be detected in washing solutions after 20 minutes incubation with paraformaldehyde (Figure 7 f). Accordingly, incubation with paraformaldehyde for at least 20 minutes reduced the titer of HTNV below the detection limit, i.e. by at least 6 log scales (Table 1).

Acetone/methanol fixation.

In general, virus-infected cells transferred onto slides for an IFA are fixed by incubation in acetone/methanol (1:1) for 10 minutes. In two independent experiments the effectiveness of the fixation procedure was verified for HTNV-infected cells. Remaining infectivity of cell lysates was determined by inoculation of the solution onto fresh cells and subsequent "infectivity" focus assay. Titration of non-treated cells revealed a titer of almost original infectivity (data not shown). After fixation with acetone/methanol for 10 minutes infectious particles could not be detected in cell lysates any longer, implying a reduction in virus titer of at least 6 log scales (Figure 7 d and Table 1).

Detergent-containing lysis buffer.

For use in Western blot analysis samples are usually boiled in a detergent-containing lysis buffer. As the remaining denaturing substances would interfere with the following titration of potentially preserved infectious particles on Vero E6 cells, an ultracentrifugation step is needed to remove residual detergents. To test whether the lysis buffer efficiently inactivates virus-infected cells, the appropriate lysates were ultracentrifugated and pellets were used for subsequent analysis.

Titration of pelleted, non-treated freeze-and-thaw-lysates (without the use of detergents) resuspended in a low-salt buffer, already showed a reduced amount of foci compared to the number of input-virions (data not shown). This reduction of infectivity can be attributed to the relatively harsh procedure of cell lysis prior to ultracentrifugation. The use of lysis buffer containing a detergent led to complete inactivation of infectious particles in the lysates, i.e. a reduction of the viral titer below detection limits (at least 3 log scales) (Figure 8 b and Table 1).

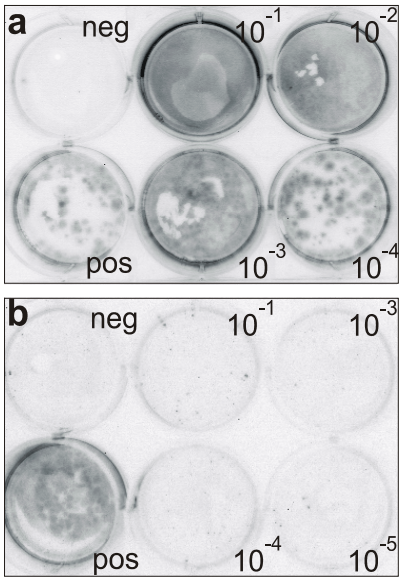

Figure 8. Inactivation of HTNV infectivity in infected cells by incubation with detergent-containing lysis buffer.

Vero E6 cells were infected with HTNV (dilutions as indicated) or left uninfected (upper left well; neg) and proteins were extracted 7 days later by detergent-containing lysis buffer (b). For comparison, an untreated virus stock was titrated on an additional plate (a). Remaining infectious particles were detected in a focus assay on fresh Vero E6 cells. An internal positive control (lower left well; pos) was added.

Taken together, the results presented demonstrate the efficient inactivation of HTNV by the chemical fixation procedures using methanol, paraformaldehyde, acetone/methanol or detergent-containing lysis buffer. The obtained samples can then be successfully subjected to various cell biological, virological and immunological investigations.

3.1.2 Infectivity of Hantaan virus can be efficiently cleared by physical forces.

UV-irradiation.

To distinguish between intracellular changes caused by replication of a virus and those only caused by adsorption and penetration without viral gene expression, inactivation of viruses by UV-irradiation has been widely used. Inactivation of HTNV stock was performed by UV-irradiation for 1, 3 and 5 minutes. Subsequently, the UV-irradiated HTNV stock and a non-treated aliquot of the same stock (Figure 9 a) were titrated in a infectivity focus assay on Vero E6 cells.

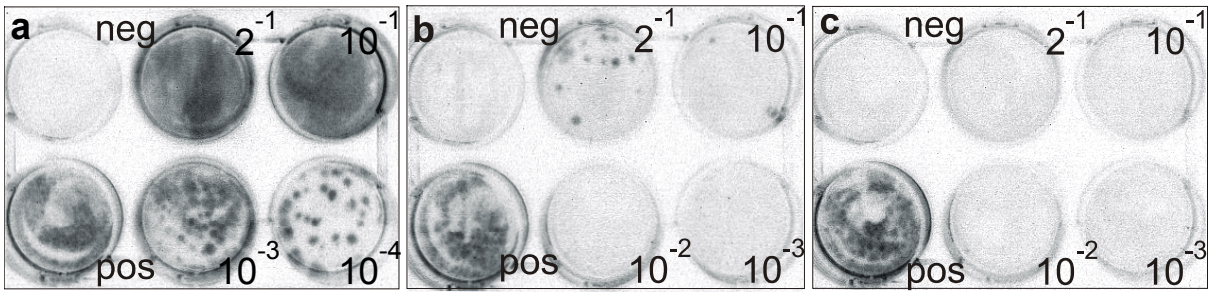

Figure 9. Dose-dependency of UV-irradiation mediated inactivation of HTNV.

HTNV stock solutions were left untreated (a) or were UV-irradiated for 1 min (b) and 3 min (c). Stock solutions were diluted (as indicated) and then screened for remaining infectivity in a focus assay on fresh Vero E6 cells. An internal negative control (upper left well; neg) contained only cell culture medium. An internal positive control (lower left well; pos) was added.

Whereas UV-irradiation for only one minute leaves remaining infectious virus (Figure 9 b), UV-irradiation for 3 minutes completely inactivated (Figure 9 c and Table 1).

Virus Filtration.

The analysis of substances, such as cytokines, secreted by infected cells into the supernatant requires the complete removal of infectious particles from the solution, preferably without affecting the biochemical properties of those molecules of interest. In this case, UV-irradiation or inactivation by chemicals might not be suitable. Therefore, filters with low protein-binding Omega™ membranes with 300 or 1,000 kDa cut-off were used to deplete virions from supernatants of infected cells.

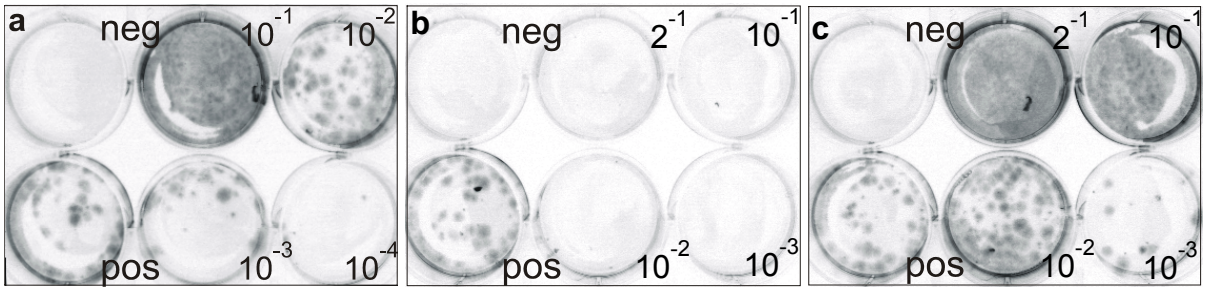

Figure 10. Detection of remaining infectivity of HTNV in supernatants of infected cells after filtration.

Supernatants of cells infected with HTNV for 7 days or left uninfected (upper left well; neg) were filtrated as described. Resulting filtrates from the use of membranes with a cut-off of 300 kDa (b) or 1,000 kDa (c) were diluted (as indicated) and screened for remaining infectivity in a focus assay on fresh Vero E6 cells. An internal positive control (lower left well; pos) was added. For comparison, an untreated virus stock was parallely titrated on an additional 6-well cell culture plate (a).

While non-filtrated supernatants of infected cells contained about 1.5×10^5 FFU/ml three days after infection (Figure 10 a), filtration with a 300 kDa cut-off membrane completely removed infectivity from the supernatant of infected cells (Figure 10 b). However, filtrating supernatants of infected cells with a 1,000 kDa cut-off membrane did not reduce the number of infectious particles (Figure 10 c).

In conclusion, physical forces such as UV-irradiation or virus filtration are appropriate means to clear HTNV-infectivity. Suchlike treated supernatants or cell lysates can be utilised in subsequent cell biological, virological and immunological investigations as has been shown during our studies.

3.2 HUVEC as an *in vitro* infection model for hantaviruses.

Since the pathogenesis of HFRS is characterised by vascular instability and viral antigen has in fact been found in endothelial cells of postmortem tissues from patients with HFRS, human umbilical vein derived primary endothelial cells (HUVEC) were chosen for the present infection studies [Peters, 99; Poljak, 94].

Although experimental infection of endothelial cells with hantaviruses has been reported previously [Pensiero, 92; Yanagihara, 90], the susceptibility of the primary endothelial cells to infection with the defined hantavirus strains used in this study was verified. To this end, HUVEC were infected with pathogenic HTNV and nonpathogenic TULV at different multiplicities of infection (MOI) and incubated over a period of up to 7 days. Cells were examined for the presence of viral antigen, both by IFA or by Western blot analysis.

3.2.1 Hantaviruses infected HUVEC *in vitro* without causing any apparent damage to the cells.

Intracytoplasmic, virus-specific granular fluorescence was readily detectable in endothelial cells 24 hours after inoculation with both hantaviruses tested, even when infected at a low MOI of 0.1 (data not shown). However, infection with higher MOI such as 1 or even 10 did not result in a drastically different effect. After three days of infection nearly all cells of the monolayer cultures contained virus antigen, independent of the amount of input-virus (Figure 11 a, d). The majority of the N protein in the infected cells was localised in the perinuclear region. This distinctive site of intracellular localisation of hantavirus antigen was observed for both HTNV and TULV-infected HUVEC. Typically, cells exhibited a filamentous staining pattern of the antigen during the first days of infection, while a punctuate pattern dominated

the staining at later time points postinfection (Figure 11 b, e). In contrast, viral antigen was not detected in uninfected HUVEC, which were included in the analysis as a negative control (Figure 11 c, f).

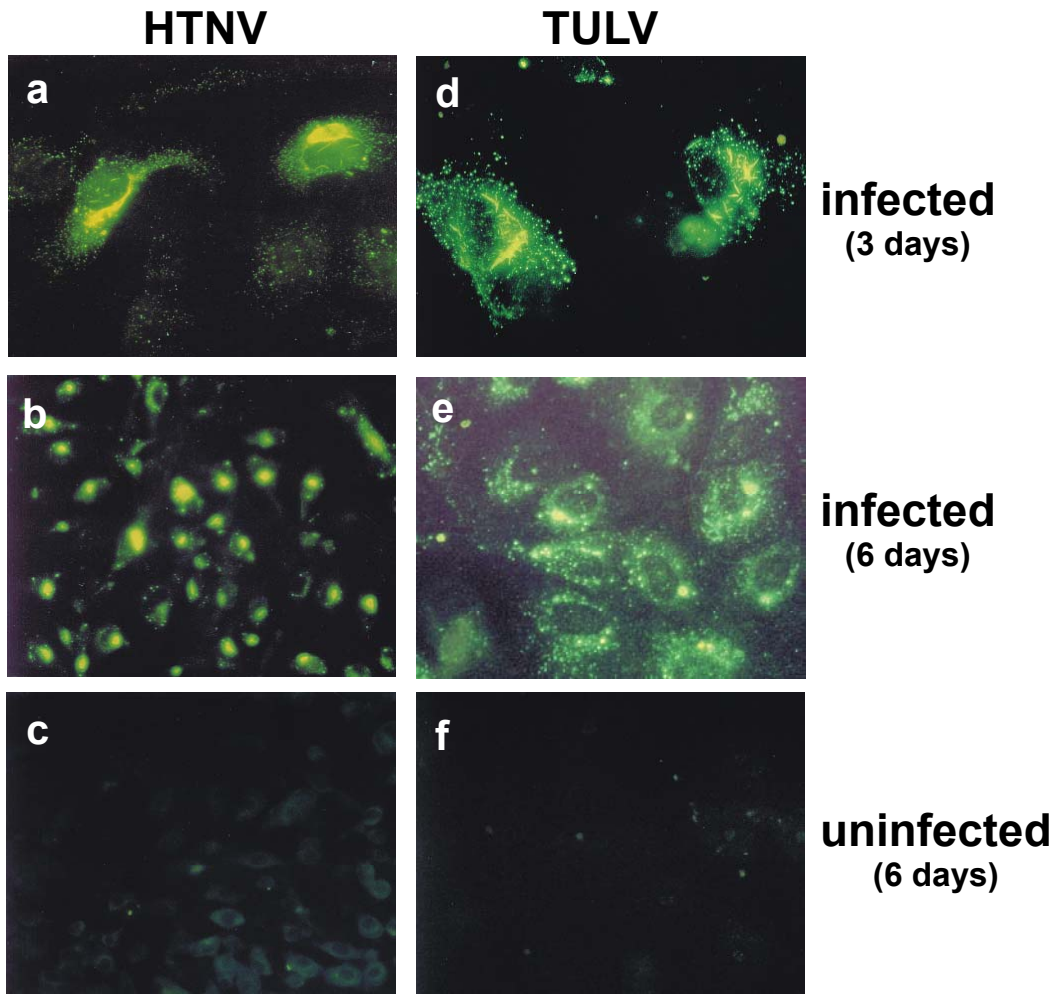

Figure 11. Detection of viral N antigen in HTNV- and TULV-infected HUVEC.

Cells were infected with HTNV or TULV at a MOI of 1. Three days postinfection the distribution of viral antigen was visualised by immunocytochemistry in TULV-infected HUVEC by using a rabbit-derived polyclonal antiserum (d) and in HTNV-infected HUVEC by employing monoclonal antibody 1C12 specific for hantavirus N protein (a). Original magnifications, ×63. After six days of infection staining of TULV antigen (e) and HTNV antigen (b) was dominated by a punctuate pattern. Original magnifications, ×63 and ×40, respectively. As a negative control uninfected HUVEC were included in the analysis (c, f). Original magnifications, ×40.

Likewise, viral N antigen could be detected by Western blot analysis as early as 24 hours postinfection in cells infected at both MOI 0.1 or 1, again using HTNV-crossreactive human antiserum or a mouse-derived monoclonal antibody (Figure 12). As expected, hantavirus antigen was undetectable in mock infected HUVEC using UV-irradiated viruses (Figure 12).

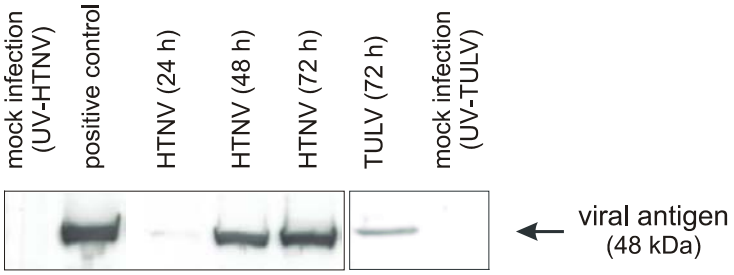

Figure 12. Expression of viral N antigen in HUVEC infected with HTNV or TULV.

HUVEC were infected with HTNV, TULV or UV-inactivated virus (mock infection) at MOI of 1. Lysates were prepared at different time points postinfection. Expression of viral antigen was analysed by Western blotting using human-derived antiserum or monoclonal antibody 1C12. Lysates of VeroE6 cells incubated for 5 days with HTNV were used as a positive control. The results shown are representative of three independent experiments with HUVEC derived from three different donors.

These results show that human endothelial cells readily support HTNV- and TULV-antigen production *in vitro*. On the basis of the morphological characteristics of viable endothelial cells cytopathic effects could never be observed by light microscopy analysis at any time point postinfection (Figure 13).

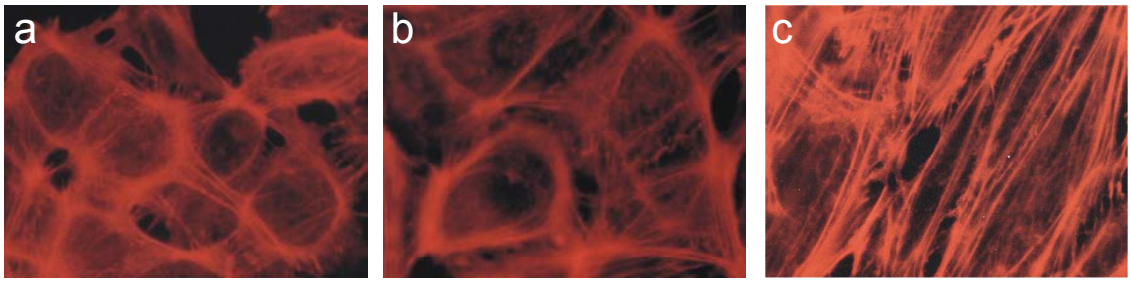

Figure 13. Staining of cytoskeletal structures in HUVEC infected with HTNV.

Staining of confluent cultures of human umbilical vein endothelial cells (HUVEC) with rhodamine-labelled phalloidin which specifically labels actin filaments, but not actin monomers. Organisation of actin microfilaments is essential for the shape of an endothelial cell (a). Stimulation with TNFα was associated with a characteristic loss of the peripheral actin band and increased intensity of actin stress fibers throughout the cell (c). Infection with HTNV (MOI 1) did not result in altered cytoskeletal structures (b).

3.2.2 Modulation of molecules involved in apoptosis was not observed in hantavirus-infected HUVEC.

The earliest indications of apoptotic cell death are morphological alterations of cells such as chromatin condensation, disappearance of the nucleolus, and alterations of the cell surface, characterised by the occurrence of blebs [Huppertz,

99]. However, such indices of apoptosis have never been observed in hantavirus-infected primary cells *in vitro*.

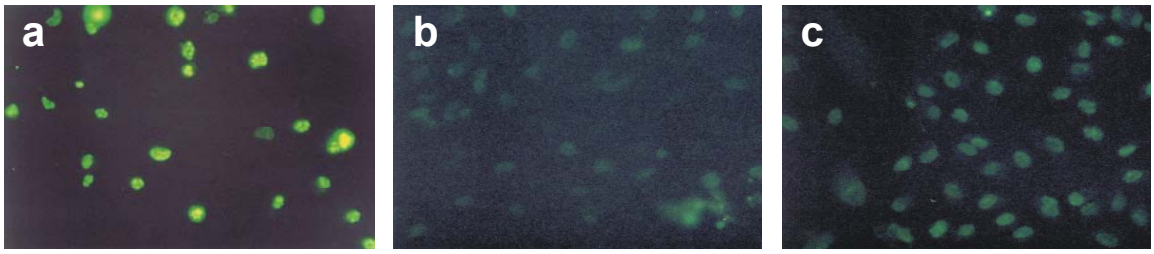

Figure 14. Apoptosis was not detected in HTNV-infected cells.

HUVEC were grown in chamber slides and infected with HTNV (MOI 1) when being confluent. At different time points postinfection DNA strand breaks were detected *in situ* using the TUNEL assay [Gavrieli, 92]. Stimulation with staurosporin induced apoptosis in HUVEC manifested as green fluorescence signals in the nucleus (a). In untreated control cells (b) as well as in HTNV-infected cells (day 3 postinfection, c) apoptosis could not be detected.

Extensive DNA degradation is a characteristic event which occurs in the early stages of apoptosis. DNA strand breaks can be detected in a TUNEL assay by enzymatic labelling of the free 3'-OH termini with modified nucleotids such as FITC-dUTP. Apoptotic cell death can then be visualised by fluorescence microscopy. To this end, HUVEC were infected with HTNV at a MOI of 1 and subjected to a TUNEL assay three and five days postinfection. Whereas stimulation of HUVEC with staurosporin for six hours strongly induced DNA strand breaking (Figure 14 a), infection with HTNV did not result in detectable levels of DNA strand breaks pointing to apoptotic cell death (Figure 14 c), nor did it in untreated control cells (Figure 14 b).

Apoptotic cell death is an active process which requires controlled gene expression. The failure of hantaviruses to induce apoptosis might be the result of specific induction of anti-apoptotic molecules such as protein kinase Akt or bcl-2.

To address the question whether hantaviruses are capable to interfere with apoptosis and thereby promote cell survival, HUVEC were infected with pathogenic HTNV at MOI of 0.1. Cell lysates were extracted at different time points postinfection and expression levels of bcl-2 protein were determined by immunoblot analysis.

HTNV-infected HUVEC did not show induction of bcl-2 protein at any time point analysed from 30 minutes up to 24 hours postinfection (data not shown), and TNFα failed to induce expression of bcl-2. Furthermore, cells infected with HTNV in the same timeframe were treated with TNFα for 30 minutes prior to cell lysis to evaluate the ability of the virus to intercept a strong apoptotic stimulus. However, a modulation

of expression levels of bcl-2 could not be detected in endothelial cells, that had been both infected with HTNV and stimulated with TNFα. Again, neither in uninfected control cells nor in TNFα treated endothelial cells, used as positive control, altered levels of bcl-2 expression were observed (Figure 15 A).

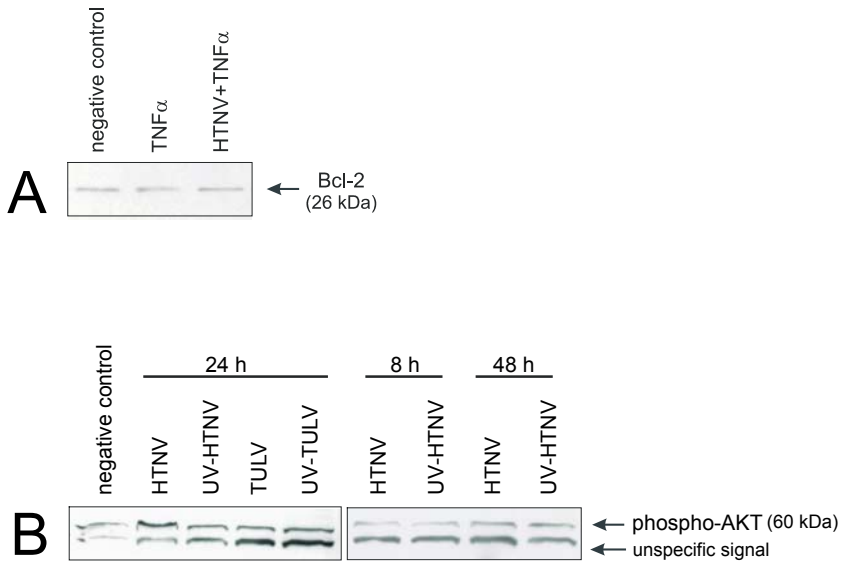

Figure 15. Expression level and activation status of proteins involved in apoptosis in response to a hantavirus infection.

(A) Cell lysates prepared after infection with HTNV (MOI 0.1) or after HTNV infection with subsequent TNFα-treatment were subjected to Western blot analysis. Expression levels of bcl-2 protein were determined at different time points postinfection using a specific bcl-2 antibody. (B) Additionally, cells were infected with HTNV or TULV (MOI 1) and proteins were extracted at different time points postinfection. Amounts of phosphorylated protein kinase Akt were determined by Western blot analysis using protein kinase Akt phosphospecific antibody. The results presented are representative of three independent experiments with cells derived from three different donors.

To assess the relevance of the protein kinase Akt in the context of apoptosis due to an infection with hantaviruses, HUVEC were infected with both pathogenic HTNV and nonpathogenic TULV at a MOI of 1. Again, proteins of infected cells were extraced by cell lysis at different time points postinfection. Activation, i.e. phosphorylation, of protein kinase Akt in response to an infection with hantaviruses was analysed by immunoblot using phospho-Akt-specific antibodies.

Levels of phosphorylated protein kinase Akt were low in uninfected or mock-infected control cells and stimulation with TNFα or IFN-β did not result in a distinct increase of activated Akt. TULV-infection did not lead to altered amounts of phosphorylated Akt in HUVEC at any time point analysed from 1 hour to three days postinfection (data not shown). Similar results were obtained for HUVEC infected with HTNV. However, after 24 hours of infection with HTNV a distinct but transient

increase of phosphorylated protein kinase Akt was detected in lysates of infected HUVEC (Figure 15 B). In contrast, HUVEC infected with HTNV for two or three days did not show elevated amounts of phospho-Akt (Figure 15 B and data not shown).

In conclusion, these experiments could not demonstrate a significant impact of programmed cell death in primary endothelial cells in the course of an infection with both pathogenic HTNV or nonpathogenic TULV. Both anti-apoptotic bcl-2 and protein kinase Akt were not induced in response to an infection with HTNV or TULV, except for a short however transient activation of protein kinase Akt after 24 h of HTNV infection. Furthermore, DNA fragmentation which would directly point to apoptosis could not be observed in HTNV-infected HUVEC.

3.2.3 Kinetics of a productive infection with hantaviruses varied between HTNV and TULV.

The presence of virus antigen in infected cells, however, does not tell us whether the virus is actually multiplying. To adress this question, medium was removed from hantavirus-infected HUVEC at different time points after infection. For comparison, Vero E6 cells, lacking type-1 IFN genes [Diaz, 88], were likewise infected with HTNV or TULV. The production and subsequent secretion of infectious virus progeny into the supernatant was determined by focus assay.

HUVEC showed production of HTNV and TULV on day one postinfection, when infected at MOI of 1. The viral titers peaked about one to three days after inoculation with the respective virus and then declined over the next days (Figure 16). However, it was observed that cells infected with the nonpathogenic TULV produced much fewer viral particles than HTNV-infected HUVEC. Two days postinfection, for instance, viral titers produced by HTNV-infected cells added up to 10^6 focus-forming units (FFU)/ml. In the supernatant of TULV-infected HUVEC, however, only 10^3 FFU/ml were detected. Endothelial cells did not produce significant levels of infectious HTNV after six to seven days of infection, whereas the level of TULV production ceased even earlier.

These results indicate that both the pathogenic HTNV and the nonpathogenic TULV could not only successfully enter HUVEC and induce viral antigen production, but were also able to efficiently replicate within these cells. However, yields of infectious HTNV were somewhat higher in HUVEC compared to TULV which

replicated far less efficiently. In fact, HTNV but not TULV creates a time window from 16 h to 48 h postinfection in which it can efficiently replicate and disseminate.

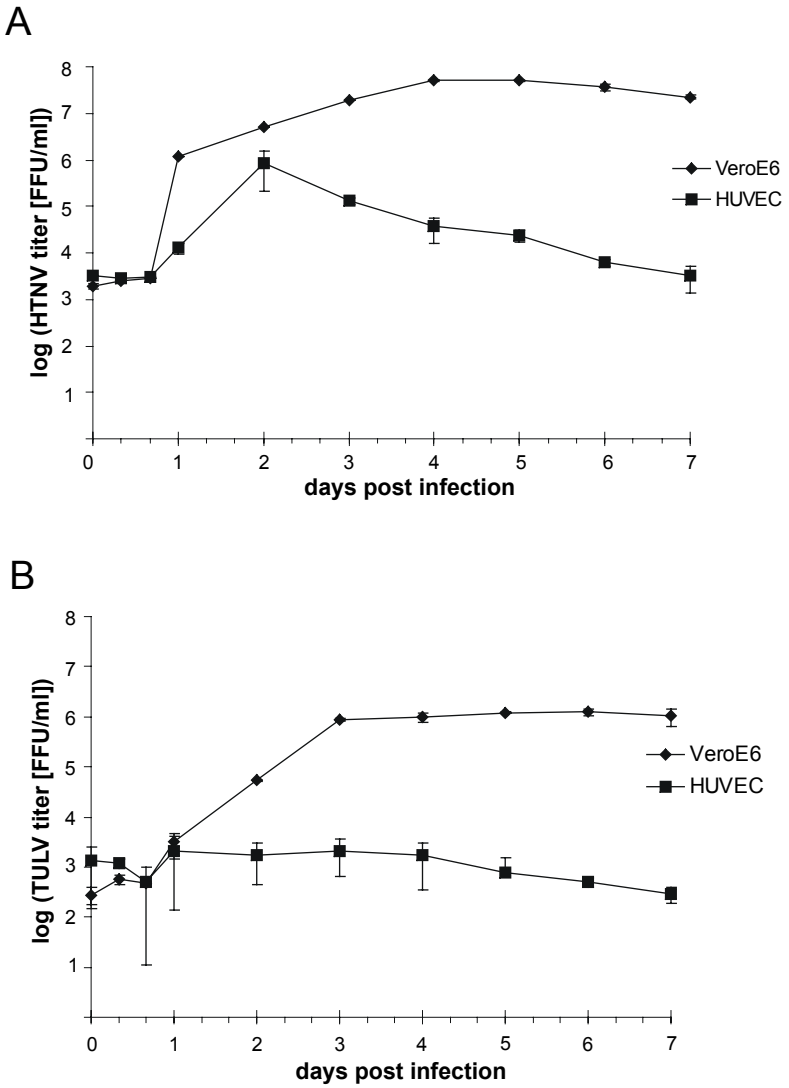

Figure 16. Kinetics of virus production in HTNV- or TULV-infected cells.

Supernatants of infected cells were collected at the time points indicated and virus titers were determined by chemiluminescence focus detection assay using human-derived anti-DOBV-positive, HTNV-cross-reactive, or anti-PUUV-positive, TULV-cross-reactive sera, respectively. Kinetics of virus production by HTNV-infected (A) and TULV-infected (B) HUVEC and VeroE6 cells are presented over a period of seven days postinfection. HUVEC derived from three different donors were used in three separate experiments. Vero E6 cells were used in two individual experiments. Titers obtained are given as mean ± SD and are shown on log scale.

Until day 2 postinfection HTNV could replicate in HUVEC nearly as efficient as in Vero E6 cells. Thereafter, viral titers produced by HTNV-infected HUVEC declined whereas viral titers produced by HTNV-infected Vero E6 cells remained high. In contrast, TULV-infected HUVEC produced only low viral titers showing no peak when

compared to TULV-infected Vero E6 cells. Both hantavirus species can efficiently grow in Vero E6 cells lacking type-1 IFN genes (Figure 16).

3.3 Intracellular responses to a hantavirus infection.

In the absence of observable cytopathology, hantavirus infection of endothelial cells may result in altered gene regulation, such as an increased expression of inflammation markers or antigen presentation molecules. Alternatively, a hantavirus infection may provoke a modulation of the activation status of intracellular signal transduction pathways. Such modulation may include phosphorylation of constitutively expressed molecules which are then available in their active version.

3.3.1 HTNV infection failed to activate MAP kinases in HUVEC.

At the molecular level, such modulation of signal transduction pathways is often achieved by activating or deactivating regulatory proteins through phosphorylation by protein kinases. To begin with, focus was put upon the intracellular pathways involved in inflammation such as the MAP kinase family. To assess whether the mitogenic p38 and p42/44 kinase cascades are activated by hantaviruses, HUVEC were infected with HTNV at different multiplicities of infection (MOI 0.1 and 1). Cells were subsequently analysed for p38 and p42/44 activity at different time points after infection.

In an intact endothelial cell both MAP kinases mentioned are constitutively expressed, but are not activated. Generally, MAP kinase p38 becomes activated and thereby phosphorylated by a variety of stress signals including UV irradiation, inflammatory cytokines, and, intriguingly, dsRNA [Iordanov, 00]. In fact, activation of MAP kinase p38 was regularly observed in HUVEC stimulated with 10 ng/ml TNFα for 10 minutes. However, after infection with HTNV phospho-p38 MAP kinase-specific bands could not be detected in Western blot analyses at any time point postinfection. The time frame analysed ranged from 15 minutes to 30 hours after inoculation with pathogenic HTNV (Figure 17). Even an infection with high viral doses (MOI 10) did not lead to an activation of MAP kinase p38 (data not shown).

Similar results were obtained for MAP kinase p42/44 (data not shown). Accordingly, activation of this proliferation and differentiation MAP kinase was not observed in HUVEC at any time point postinfection, even when HUVEC were infected at high MOI. The efficient infection of cells was randomly proven by detection of viral antigen in an additional Western blot analysis (Figure 12).

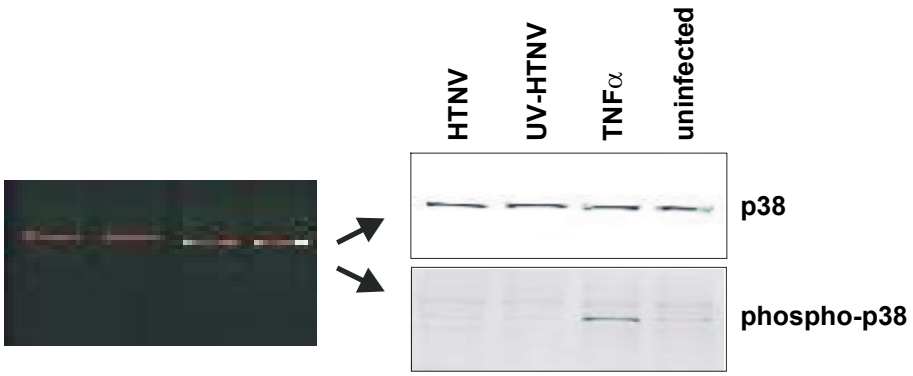

Figure 17. Analysis of phosphorylated MAP kinase p38 in HUVEC after infection with HTNV.

After infection with HTNV or TULV (data not shown) at MOI 1 cell lysates were extracted at different time points ranging from 15 minutes to 30 hours postinfection. Phosphorylation of MAP kinase p38 was demonstrated by Western blot analysis using a p38 phosphospecific antibody and a Cy5.5–labelled secondary antibody. TNFα treatment of cells served as positive control. Parallel staining of the Western blot membrane using an anti-p38 antibody, which recognises all forms of p38, and a IRDye 800–labelled secondary antibody, demonstrated that the overall levels of p38 as well as the amount of loaded protein did not fluctuate. Separate experiments were conducted with cells derived from three different donors. On the left side images are displayed in colour with both colour channels overlaid, whereas greyscale images are separately shown on the right side.

These findings indicate that infection of endothelial cells with both pathogenic HTNV or nonpathogenic TULV does not induce activation of the highly conserved cytosolic serine/threonine protein kinases p38 or p42/44. Both hantaviruses evidently do not belong to the stress factors known to activate signalling events involved in inflammatory responses.

3.3.2 Analysis of potential NFκB activation and IκB-α degradation in HUVEC after infection with hantavirus.

Since an inflammatory response to a hantavirus infection on the level of MAP kinases could not be revealed, it seemed obvious, to analyse a more downstream component of signalling pathways involved in inflammation.

Activation of the transcription factor NFκB has been reported to occur rapidly in response to an extremely wide range of stimuli, including cytokines, physical stress or viral proteins. Two different approaches are applicable to examine the activation of NFκB: its translocation into the nucleus on the one hand, and the degradation of its inhibitor IκB-α on the other hand. This inhibitor is a primary target of signalling pathways which are activated by inflammatory stimuli, and which eventually result in its phosphorylation and subsequent proteolytic degradation. Once released from its inhibitor NFκB readily translocates into the nucleus.

Hantavirus infection did not cause translocation of NFκB into the nucleus.

We used a polyclonal anti-NFκB antibody and fluorescence microscopy to evaluate NFκB activity in HUVEC. Strong fluorescent signals were observed in the cytoplasm of untreated endothelial cells (Figure 18 a). After treatment with 100 ng/ml TNFα for 15 minutes cytoplasmic fluorescence decreased while nuclear fluorescence increased (Figure 18 c). This clearly demonstrated the translocation of activated NFκB protein from the cytoplasm into the nucleus in response to this proinflammatory cytokine. However, an infection with HTNV at a MOI of 0.1 did not lead to an activation of NFκB at any time point analysed, ranging from 15 minutes to 48 hours postinfection. Accordingly, fluorescence was predominantly detected in the cytoplasm of infected HUVEC (Figure 18 b).

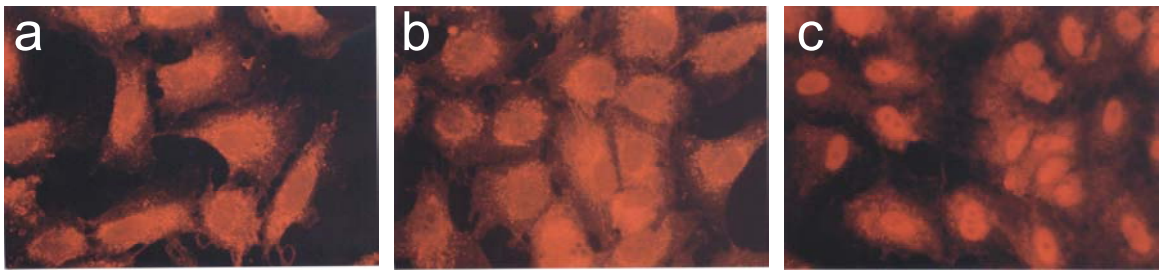

Figure 18. Cellular localisation of NFκB after an infection with HTNV.

Cells were infected with HTNV (MOI 0.1) and the cellular localisation of NFκB was visualised by immunofluorescence microscopy using NFκB-specific antibody. TNFα induced rapid translocation of NFκB p65 from the cytoplasm to the nucleus in HUVEC (c). NFκB nuclear localisation could not be observed in untreated (a) or HTNV-infected cells (b).

Degradation of IκB-α could not be detected after infection with hantavirus.

The steady state level of cytoplasmic IκB-α protein was measured by Western blot analysis, using a polyclonal anti-IκB-α antibody.

In cell extracts of untreated HUVEC consistent amounts of IκB-α could be detected, whereas stimulation of HUVEC with 10 ng/ml TNFα lead to a rapid proteolysis of IκB-α as virtually all the IκB-α present in the cytoplasm was degraded within 15 minutes. Infection with both pathogenic HTNV or nonpathogenic TULV at MOI of 1 did not lead to a change in the amount of IκB-α detected in the cell extracts at any time point analysed (Figure 19 A).

However, a viral infection might have as well an impact upon the cellular reaction to a stimulation with TNFα. A stabilisation of complexed IκB-α by the virus

could, for instance, suppress or postpone inflammatory processes in response to the viral infection. To adress this question, HUVEC were infected with HTNV (MOI 1) and subsequently stimulated with 10 ng/ml TNFα.

Treatment of HTNV-infected cells with TNFα, though, led to the usual degradation of cytoplasmic IκB-α. No difference between infected or uninfected cells could be detected concerning IκB-α degradation after TNFα-stimulation (Figure 19 B).

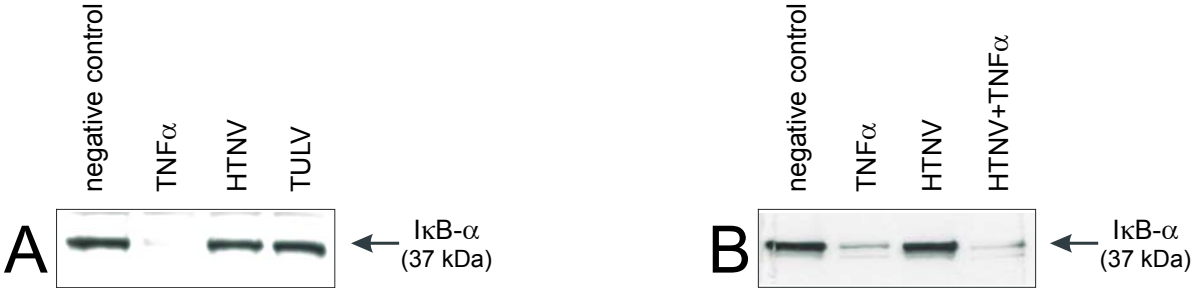

Figure 19. Degradation of cytoplasmic IκB-α after HTNV or TULV infection.

(A) After infection of HUVEC with HTNV or TULV (MOI 1) cell lysates were prepared at different time points ranging from 10 min to 30 h postinfection. For negative control cells were left uninfected. Treatment with TNFα was used as a positive control. (B) Additionally, cells were infected with HTNV and subsequently treated with TNFα. By Western blot analysis using IκB-α antibody levels of cytoplasmic IκB-α were detected. 50 μg of cytoplasmic protein were loaded on all lanes. Cells derived from three different donors were subjected to Western blot analyses in three independent experiments and representative results are presented.

Thus, infection with pathogenic HTNV as well as nonpathogenic TULV does not lead to activation of central transcription factor NFκB. Likewise, a modulation of its inhibitor IκB-α could not be observed after infection.

3.4 Modulation of immunoregulatory proteins after a hantavirus infection.

Innate immunity represents the first line of cellular defence against invading pathogens, including viruses, before a complex adaptive immune response is launched against such pathogens. One of the principal features of innate immunity is activation of the already mentioned NFκB transcription factor. Genes activated by NFκB play an important role in both adaptive (cytokines, adhesion molecules and HLA class I molecules) and innate (cytokines such as IFN) immune response.

Although activation of NFκB was not observed in the prior experiments, modulation of molecules involved in both the innate as well as in the adaptive

immune response, namely adhesion molecules, antigen presentation molecules and IFN, was investigated. A weak induction of this central transcription factor might have been overlooked or, alternatively, NFκB-independent gene transcription might have occurred.

3.4.1 Hantaviruses slightly upregulated levels of adhesion molecules.

Cell adhesion molecules are central factors for leukocyte adherence and the propagation of inflammatory responses. In fact, recognition of and adhesion between T lymphocytes and infected target cells can be enhanced by ICAM-1 and VCAM-1.

To compare such cellular responses endothelial cells were infected at a MOI of 1 by pathogenic HTNV or nonpathogenic TULV. Expression levels of these cell surface molecules were then visualised by flow cytometry analysis at different time points postinfection (Figure 20).

A slight though consistent increase in levels of ICAM-1 was observed on the surface of HUVEC infected with pathogenic HTNV or nonpathogenic TULV over six days (Figure 20 B), whereas such an upregulation could not be detected for VCAM-1 (data not shown). The effect was not observed after mock-infection of HUVEC with UV-inactivated hantaviruses. Uninfected HUVEC treated with 10 ng/ml TNFα for 24 hours, however, exhibited a strong upregulation of both ICAM-1 and VCAM-1 three days after stimulation (data not shown).

Taken together, these results do not reveal overall distinctly increased expression levels of cell adhesion molecules VCAM-1 or ICAM-1 in response to an infection with hantaviruses HTNV or TULV. Moreover, cell surface ELISA technique could not demonstrate an induction of adhesion molecules such as ICAM-1 or E-selectin after infection with HTNV (data not shown).

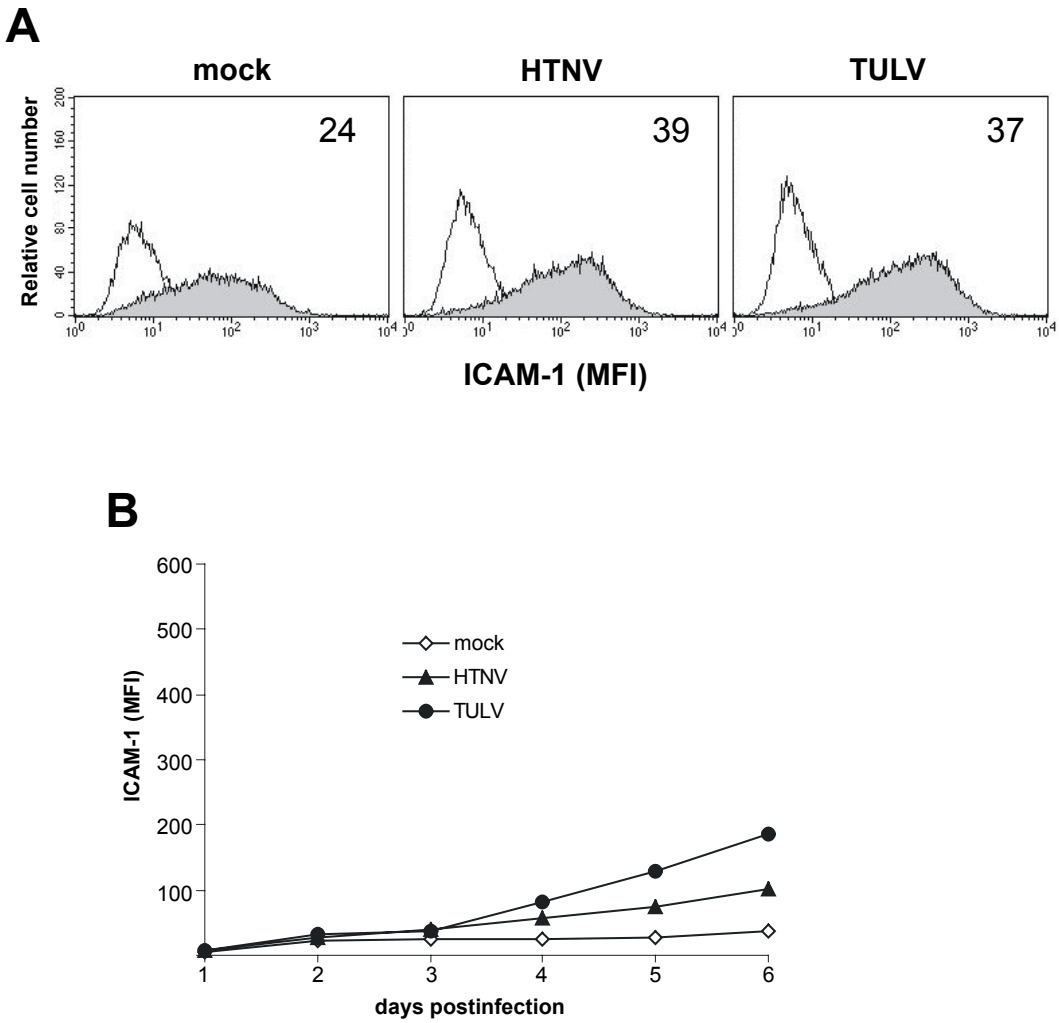

Figure 20. Expression levels of ICAM-1 on HUVEC after infection with HTNV or TULV.

HUVEC were infected with HTNV or TULV (MOI 1). Infection with UV-irradiated HTNV was included as mock-infection. Expression levels of ICAM-1 were evaluated by flow cytometry analysis. (A) Unfilled curves show staining of cells with an isotype control antibody, whereas grey filled-in curves represent expression of ICAM-1 at day three postinfection. Mean fluorescence intensity (MFI) values given in the upper right corner of each histogram are representative for three separate experiments with cells derived from three different donors. (B) Temporal pattern of ICAM-1 expression on HTNV- or TULV-infected HUVEC. The y-axis shows the MFI at day 1 to 6 postinfection as indicated. One representative experiment out of five is depicted.

3.4.2 Hantaviruses modulated antigen presentation molecules.

Apart from the above-mentioned cell adhesion molecules, additional molecules on the surface of infected cells are important for the functioning of antiviral T lymphocytes.

In fact, both CD4- and CD8-positive T lymphocytes have to interact with antigen presenting molecules in order to gain functional activity and to monitor infection. Recognition of antigen-presenting molecules, namely HLA class I, on the surface of an infected cell by cytotoxic CD8-positive T lymphocytes will eventually result in the

elimination of such virus-infected cells. Activation of helper CD4-positive T lymphocytes relies on the interaction with HLA class II molecules to support the elimination of virus-infected cells. HLA class II molecules are usually not expressed on endothelial cells. However, IFN-γ is able to induce such unusual expression pattern and thereby promotes the recognition of infected cells by helper T lymphocytes.

Neither HTNV- nor TULV-infected HUVEC expressed HLA class II molecules at any time point analysed. In contrast, HUVEC treated with IFN-γ showed clearly increased expression levels of HLA class II molecules on their surface (Figure 21).

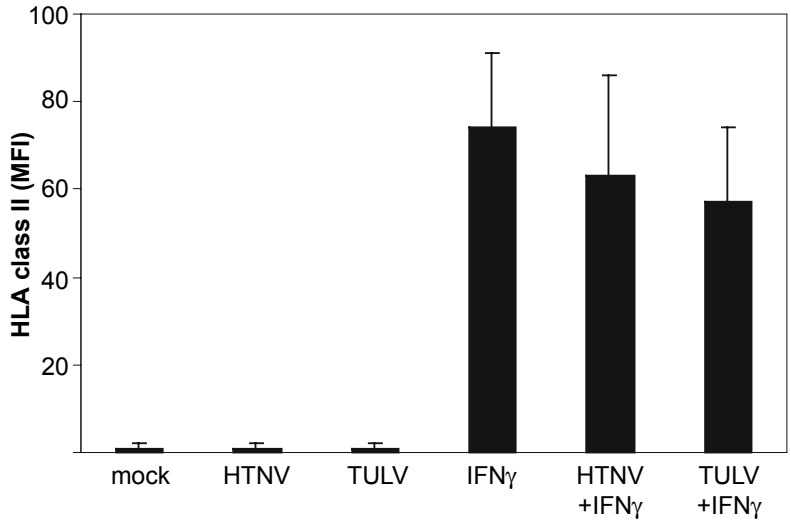

Figure 21. HLA class II-expression on HUVEC in response to an infection with HTNV or TULV.

Cells were infected with HTNV or TULV (MOI 1) and stained for HLA class II molecules three days after infection. Mock-infected cells were included as a control. Stimulation with IFN-γ induces the upregulation of HLA class II in endothelial cells and served as positive control. In order to detect synergistic effects infected cells were simultaneously stimulated with IFN-γ as indicated. Expression levels of HLA class II molecules were quantified by flow cytometry. Mean fluorescence intensity values (MFI) are given as mean ± SD of three individual experiments with cells derived from three different donors.

Both HTNV and TULV strongly augmented the expression of HLA class I molecules on the surface of HUVEC at day 3 postinfection (Figure 22 A). Kinetic analyses revealed that TULV-infected HUVEC more rapidly upregulated HLA class I molecules with a peak at day 2 to 3 postinfection. In contrast, HTNV-infected HUVEC more gradually increased expression of HLA class I molecules reaching peak levels at day 4 to 5 postinfection. These phenotypic changes were not observed after mock-infection of HUVEC with UV-inactivated hantaviruses (Figure 22 B).

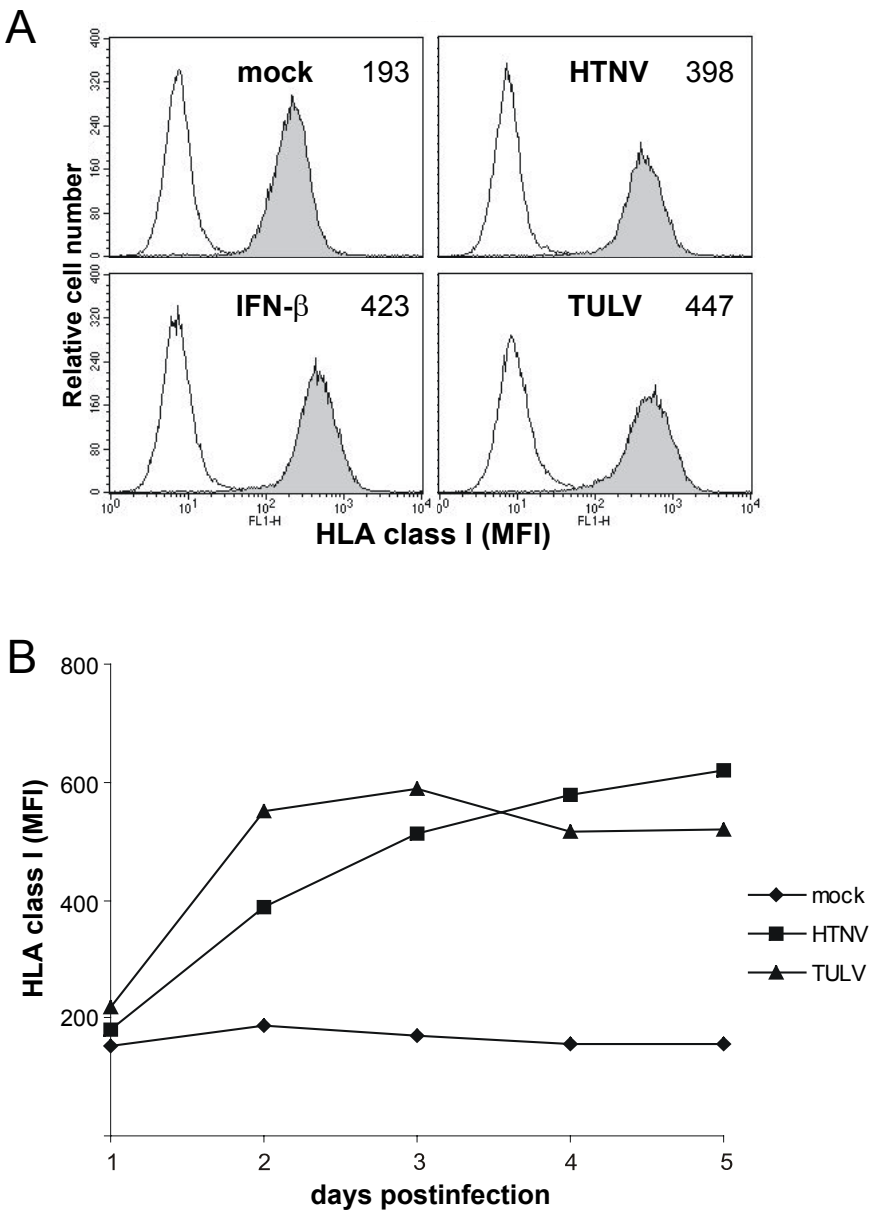

Figure 22. Temporal pattern of HLA class I expression on HTNV- or TULV-infected HUVEC.

HUVEC were infected with HTNV or TULV (MOI 1). UV-irradiated HTNV was used for mock-infection. (A) Histograms are showing HLA class I expression at day 3 postinfection as indicated. As a positive control HUVEC were treated with IFN-β (20 000 U/ml for 24 h). Grey filled-in curves represent expression of HLA class I molecules, whereas unfilled curves show staining of cells with an isotope control antibody. On the x-axis, the fluorescence intensity (log scale, 4 decades) is shown, the y-axis depicts the relative cell number. Mean fluorescence intensity (MFI) values are depicted in the upper right corner of each histogram. The results shown are representative of eight separate experiments with cells derived from eight different donors. (B) The kinetics of HLA class I expression on HTNV- and TULV-infected HUVEC were analysed. The y-axis shows the MFI at day 1 to 5 postinfection as indicated. One representative experiment out of eight is depicted.

An infection with a low virus dose such as MOI of 0.1 did not result in increased expression levels of HLA class I molecules on the surface of HUVEC. However, augmented expression levels of HLA class I molecules could be detected

reproducibly after infection with hantavirus at MOI of 1 or 10 (Figure 23). Apparently, the MOI used has a strong impact upon the results obtained in the studies described here. Conclusions drawn from infection experiments using a low MOI of 0.1 do not necessarily hold true for experiments conducted at higher MOI such as 1 or even 10. In fact, the modulation of antigen presentation molecules on HUVEC was clearly dose-dependent.

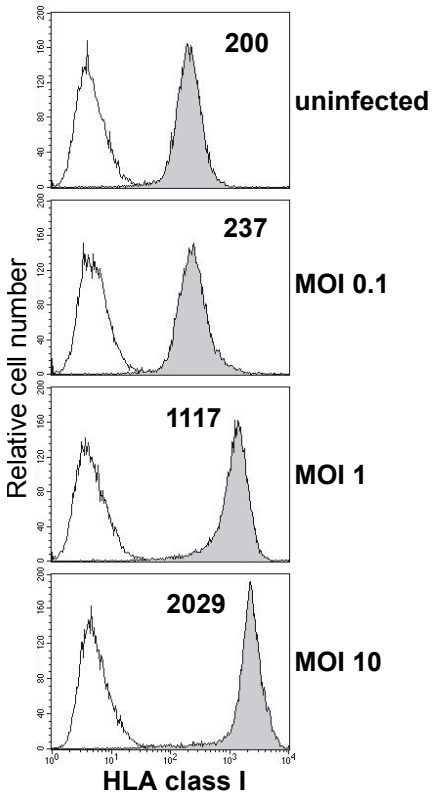

Figure 23. Influence of viral load on expression levels of HLA class I molecules on endothelial cells.

HUVEC were left uninfected or infected with HTNV at different MOI (0.1, 1 or 10). At day 3 postinfection expression levels of HLA class I molecules were determined by flow cytometry analysis. Grey filled-in curves represent expression levels of HLA class I molecules whereas unfilled curves show staining of cells with an isotope control antibody. On the x-axis, the fluorescence intensity (log scale, 4 decades) is shown. The y-axis depicts the relative cell number. The mean fluorescence intensity (MFI) is given in the upper right corner of each histogram. The results shown are representative of three separate experiments with cells derived from three different donors.

3.4.3 Production of IFN was adjusted by hantaviruses.

The innate immune response, including the production of cytokines, represents a hallmark of the host defence against viral infections. The antiviral cytokine IFN-α/β (type I IFN), produced following viral infection, exerts an antiviral state by inducing

the Janus kinases/signal transducer and activator (JAK-STAT) pathway. IFN-γ (type II IFN) increases the efficiency with which target cells present viral antigen in association with HLA class I molecules for recognition by cytotoxic T lymphocytes. Moreover, IFN-γ induces the expression of HLA class II proteins on the surface of cells that do not normally express them. Thereby such cells, like endothelial cells, are enabled to present antigen to helper T lymphocytes.

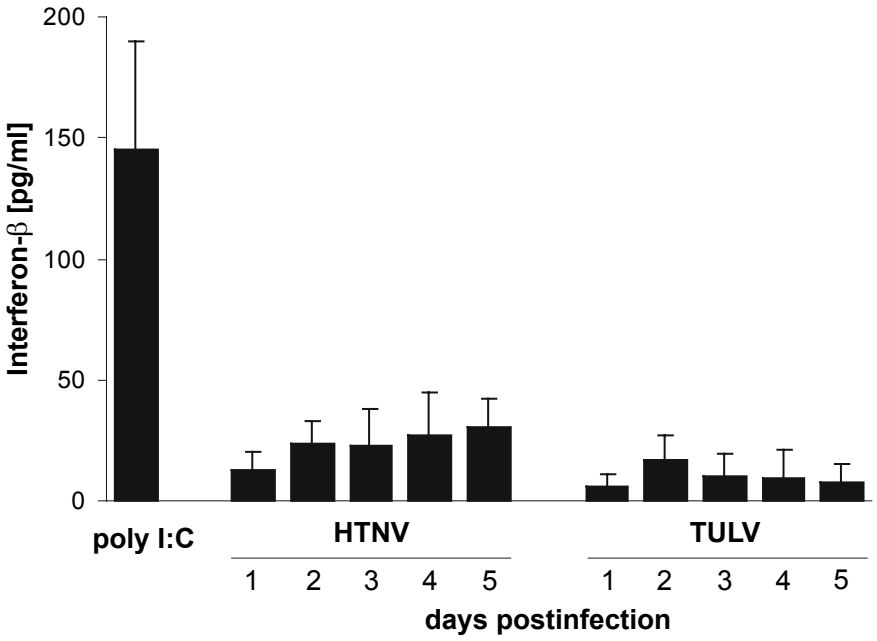

Figure 24. Production of IFN-β by endothelial cells after infection with HTNV or TULV.

ELISA technique was used to analyse supernatants from infected HUVEC (MOI 1) collected at the time points indicated. As a positive control supernatant from cells treated with poly I:C (10 μg/ml for 24 h) was included. Supernatants from mock infected cells served as negative control (data not shown). Concentrations (pg/ml) are given as mean ± SD of three individual experiments with cells derived from three different donors.

To assess the role of type I and type II IFN during a hantavirus-infection their synthesis was evaluated in HUVEC infected with pathogenic HTNV or nonpathogenic TULV at a MOI of 1 at different time points postinfection.

As a positive control HUVEC were treated with poly I:C, a synthetic mimic of double-stranded RNA. HUVEC treated with poly I:C for 24 hours released high amounts of IFN-β whereas infected cells produced much less cytokine as determined by ELISA technique. In the supernatant derived from HTNV-infected HUVEC consistently higher amounts of IFN-β were found than in supernatant collected from

cultures of TULV-infected HUVEC (Figure 24). Neither HTNV nor TULV induced HUVEC to secrete IFN-α (data not shown).

Additionally, IFN production was assessed in a bioassay that measured antiviral activity in the supernatant of infected cells. Again, an antiviral activity was detected in supernatants of poly I:C treated control cells and HTNV-infected but was barely detectable in the supernatant from TULV-infected HUVEC (Figure 25).

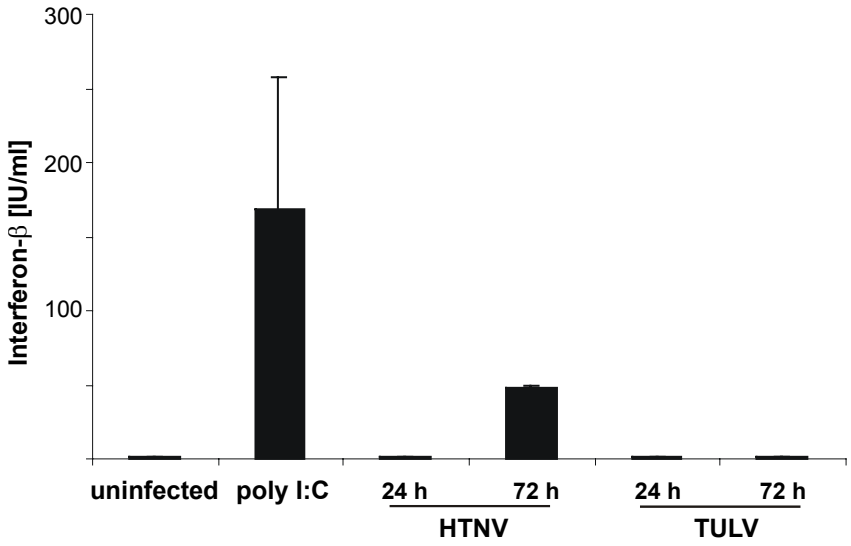

Figure 25. Detection of antivirally active IFN in the supernatant of hantavirus-infected HUVEC.

A bioassay as described in materials and methods was employed to determine the concentration of IFN-β in supernatants collected from infected HUVEC (MOI 1) at the time points indicated. Uninfected cells served as a negative control. Stimulation with poly I:C strongly induces production of IFN and was used as a positive control. Concentrations are given as mean ± SD of two independent experiments with cells derived from two different donors.

Production of IFN type I molecules was also monitored on the mRNA level by quantitative real-time RT-PCR technique. This type of analysis revealed that HTNV increases the production of IFN-β mRNA postinfection starting at day 2, reaching peak levels at day 4 and decreasing thereafter. In comparison, TULV-infected HUVEC produced only low amounts of IFN-β transcripts whereas mock-infection (UV-HTNV) had no effect (Figure 26). Neither HTNV- nor TULV-infected HUVEC showed induction of IFN-α, IFN-γ or IFN-λ, a recently discovered type I IFN, gene transcription (data not shown).

Taken together, these findings suggest that HTNV elicits a stronger IFN-β response than TULV most likely due to more efficient replication.

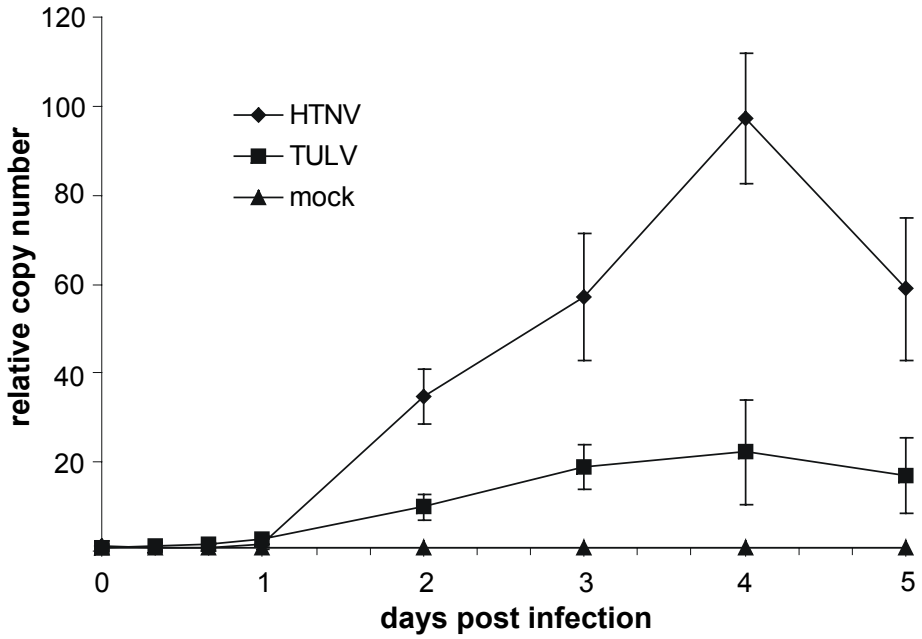

Figure 26. Quantification of IFN-β transcripts in HUVEC in response to an infection with HTNV or TULV.

Quantitative real-time RT-PCR analysis was performed to determine the relative copy number of IFN-β transcripts in HUVEC in response to an infection with HTNV or TULV (MOI 1). As a positive control cells were stimulated with poly I:C and subjected to the same analysis. UV-inactivated HTNV was used for mock infection. RNA was isolated at the time points indicated. The results depicted are given as mean ± SD and are derived from three individual experiments with cells from three different donors.

HTNV and TULV differentially regulated gene expression associated with IFN type I production.

This differential antiviral response observed in HTNV- or TULV-infected HUVEC was mirrored in the expression patterns of interferon regulatory factor-3 (IRF-3) and interferon regulatory factor-7 (IRF-7), transcription factors involved in establishing an innate antiviral state. IFN type I is induced in a virus-infected cell as a result of activation of NF-κB or IRF-3 or both. IRF-7, induced by IFN-β, then triggers the induction of different IFN genes (Figure 27).

Production of IRF-3 and IRF-7 was therefore measured in hantavirus-infected HUVEC. Again, poly I:C treated endothelial cells were used as a positive control showing increased levels of both IRF-3 and IRF-7 transcripts by quantitative real-time RT-PCR technique (Figure 28).

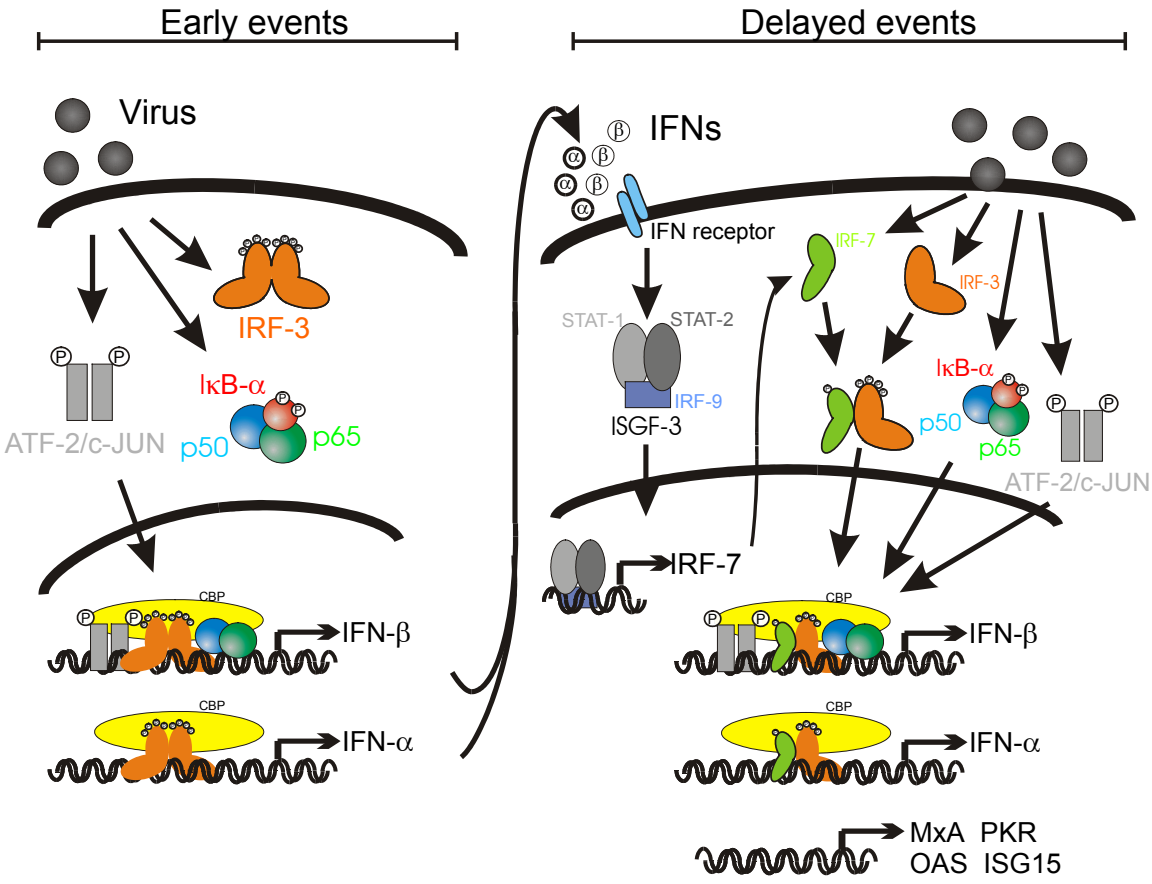

Figure 27. Early and delayed events in the IFN system.

In response to virus infection a number of signal transduction pathways are activated, ultimately leading to the activation of transcription factors that regulate immediate early genes, among which are the genes that encode type I IFN. Once secreted, IFN interact with a specific receptor at the surface of surrounding cells to induce the JAK/STAT signalling pathway, resulting in the activation of the IFN-stimulated gene (ISG) transcription factor (ISGF)-3, transcriptionfactor ATF-2/c-Jun, and the production of interferon regulatory factor (IRF)-7. Upon virus infection, IRF-3, IRF-7 and CREB-binding protein (CBP) contribute to the expression and amplification of the IFN response by inducing delayed type I IFN genes and genes that result in an antiviral state. From:Grandvaux et al., 2002 (modified).

These findings demonstrate that pathogenic and nonpathogenic hantaviruses are in fact different with regard to the virus-induced IFN response in endothelial cells. HTNV induced synthesis of IFN-β which was barely detectable after an infection with TULV. However, neither HTNV nor TULV induced significant levels of IFN-α or IFN-λ. In HTNV-infected HUVEC strongly enhanced levels of IRF-3 and IRF-7 transcripts were detected. However, this upregulation was observed to a far lesser extent in TULV-infected HUVEC.

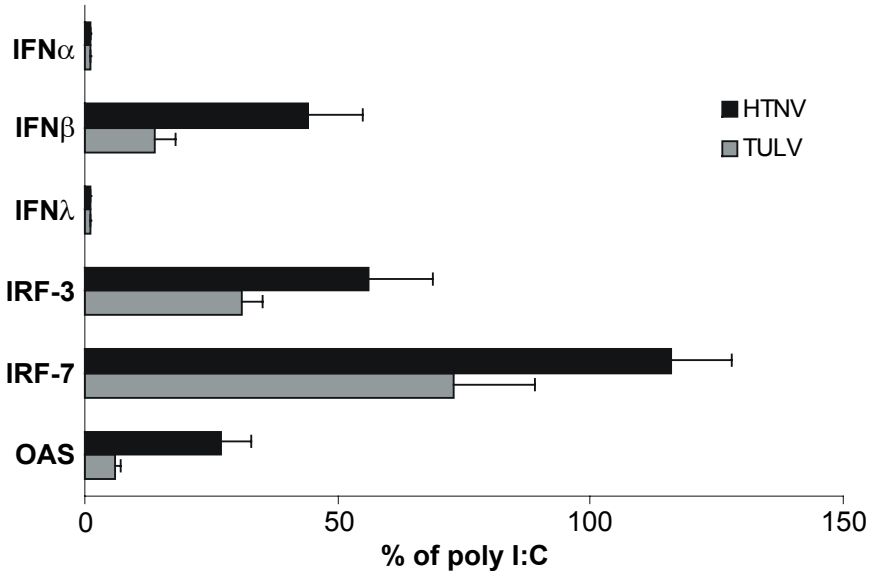

Figure 28. Transcription patterns of IFN response genes induced in HUVEC after hantavirus infection.

RNA was isolated from HUVEC three days after infection with HTNV or TULV (MOI 1). Subsequently real-time RT-PCR analysis was performed to measure transcript levels of IFN and interferon regulatory factors (IRF)-3 and -7 as well as 2´-5´-oligoadenylate synthetase (OAS). Values are given as percentage of the maximal expression levels induced by poly I:C which served as positive control stimulus. UV-inactivated HTNV was used for mock infection (data not shown). The results depicted are mean ± SD derived from three independent experiments with cells from three different donors.

These findings demonstrate that pathogenic and nonpathogenic hantaviruses are in fact different with regard to the virus-induced IFN response in endothelial cells. HTNV induced synthesis of IFN-β which was barely detectable after an infection with TULV. However, neither HTNV nor TULV induced significant levels of IFN-α or IFN-λ. In HTNV-infected HUVEC strongly enhanced levels of IRF-3 and IRF-7 transcripts were detected. However, this upregulation was observed to a far lesser extent in TULV-infected HUVEC.

3.4.4 Increased expression of IFN-inducible proteins was observed in response to hantavirus infection.

IFN as antiviral and antiproliferative cytokines mediate their effects mainly through well-conserved signalling pathways. Major molecular players involved in transducing antiviral IFN signals, namely molecules of the signal transducer of activation and transcription (STAT) family and MxA, were investigated.

HTNV but not TULV induced upregulation of STAT-1.

IFN type I signalling is furthermore mediated by phosphorylation of STAT-1. Such activated STAT molecules translocate into the nucleus and eventually activate IFN-stimulated antiviral genes. Diverse protein kinases, including the already mentioned MAP kinases, phosphorylate STATs and with it potentiate the primary STAT-activating stimulus which induces phosphorylation and subsequent dimerisation necessary for the activity as transcription factor [Aaronson, 02].

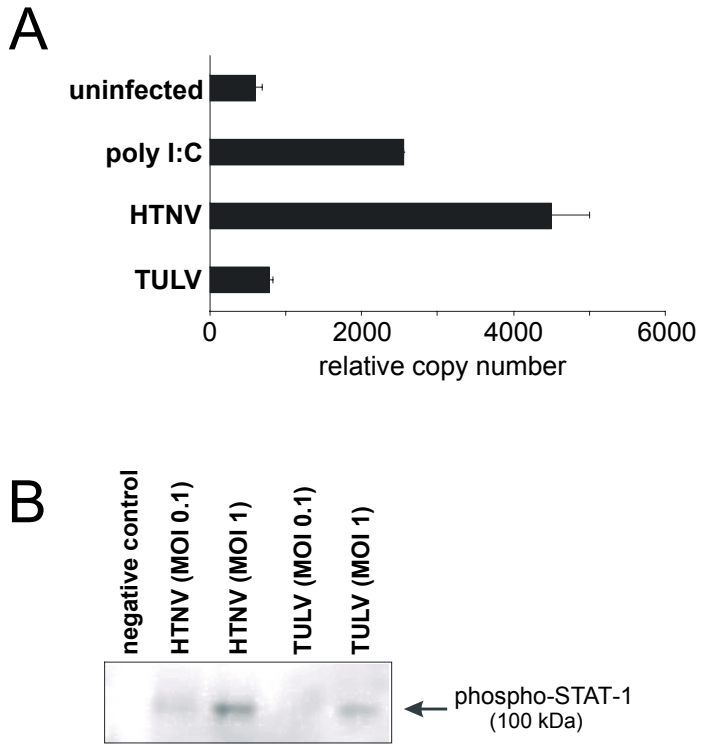

Figure 29. Production and activation status of STAT in HUVEC after infection with hantaviruses.

(A) HUVEC were infected with HTNV or TULV (MOI 1). Three days after infection RNA was isolated from cells and subjected to real-time RT-PCR analysis to quantify transcript levels of STAT-1. Stimulation with poly I:C (10 μg/ml for 24 h) served as positive control. The results depicted are relative copy numbers with mean ± SD derived from three independent experiments with cells from thee different donors. (B) Lysates of HTNV- or TULV-infected cells (MOI 0.1 or 1) were prepared three days postinfection. UV-inactivated HTNV was used for mock infection (negative control). Expression levels of phosphorylated STAT-1 were analysed by Western blot technique using phospho-STAT-1 specific antibodies.

HTNV-infection strongly induced the transcription of the STAT-1 gene three days postinfection, whereas TULV had only a marginal effect at this time point. In fact, the observed induction of STAT-1 protein in HTNV-infected cells was more pronounced compared to a positive control, comprising HUVEC which had been

stimulated with poly I:C for 24 h prior to the analysis (Figure 29 A). Western blot analysis of cell lysates obtained from HTNV- or TULV-infected HUVEC verified these findings (Figure 29 B). An infection of HUVEC with both HTNV or TULV at a low MOI (0.1) did not result in a distinct upregulation of STAT-1. However, infection at a higher MOI (1) led to clearly detectable levels of STAT-1 activation in infected HUVEC. Again, activation of STAT-1 was more pronounced in HTNV-infected HUVEC in comparison to TULV-infected HUVEC (Figure 29 B).

These findings demonstrate quantitative differences in endothelial cells concerning transcriptional and activation responses of STAT-1 between pathogenic HTNV and nonpathogenic TULV.

HTNV and TULV induced MxA protein with different kinetics.

The network of genes stimulated by IFN type I includes MxA, another protein with direct antiviral activity. MxA inhibits the growth of hantaviruses by interfering with the viral replication.

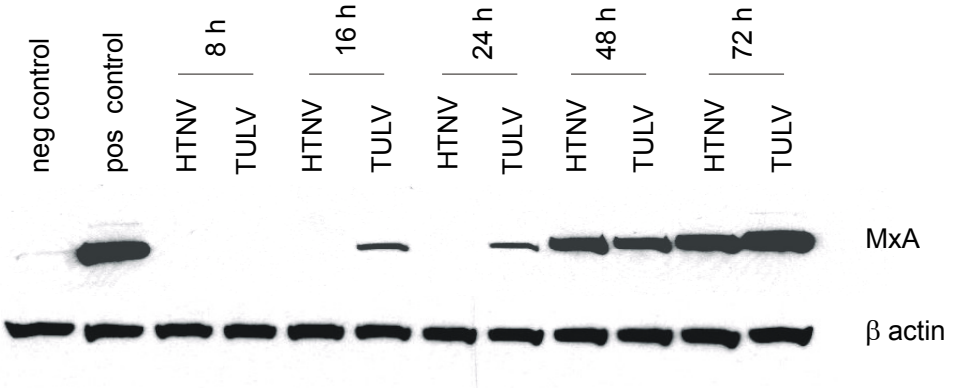

Figure 30. Kinetics of MxA protein expression in HUVEC infected with HTNV or TULV.

Cells were infected with HTNV or TULV (MOI 1) and lysates were prepared at different time points as indicated. Western blot technique was used to evaluate expression of MxA protein. Lysates of cells incubated for three days in the absence of virus served as a negative control and lysates of cells stimulated with poly I:C was used as a positive control. Expression of β-actin was determined to control the proper loading of SDS-PAGE gels. The results shown are representative of three independent experiments with cells derived from three different donors.

Thus, induction of MxA protein is an important antiviral parameter which could determine the outcome of infections with different hantaviruses. Expression levels of this large GTPase were evaluated by immunoblot in cell lysates of hantavirus-infected endothelial cells. In comparison to HUVEC treated with poly I:C, which led to a rapid induction of MxA, HTNV-infected HUVEC showed onset of MxA expression

only 48 hours postinfection. In contrast, in TULV-infected HUVEC the MxA protein was already detectable at 16 hours postinfection (Figure 30).

These results indicate that HTNV and TULV induce MxA protein with different kinetics and could explain the reduced replication rate of TULV in endothelial cells.

3.4.5 Indirect viral effects are for the most part responsible for cellular effects contributing to hantavirus-induced pathogenesis.

The question arose whether the observed upregulation of HLA class I molecules on hantavirus-infected HUVEC was caused by the virus itself or rather by a collateral mediator. In general, soluble factors, for instance, could be secreted by cells in response to a viral infection and subsequently affect neighbouring cells. Alternatively, the virus itself, the mere process of infection or viral proteins could directly provoke a modulation of the protein expression pattern in the infected cell.

To address the question whether hantaviruses primarily affect endothelial cells by means of triggering cellular IFN-β-secretion or whether a viral protein is directly triggering the cellular events observed in cell culture, two experimental approaches were chosen.

Virus-free supernatants of infected HUVEC induced HLA class I upregulation.

After three days of inoculation with HTNV (MOI 1) supernatants of infected HUVEC were filtrated. Filters were chosen to be tight enough to retain the virus and porous enough to let pass potentially secreted molecules. Virus-free filtrates were then used to stimulate untreated HUVEC and HLA class I expression levels were measured by flow cytometry another three days later. A distinct upregulation of HLA class I molecules could be detected on HUVEC directly infected with HTNV for three days (MOI 1) or stimulated with TNFα. As expected, uninfected control HUVEC did not show increased levels of HLA class I molecules. A stimulation with virus-free supernatant of infected HUVEC led to slightly augmented expression levels of HLA class I molecules on the surface of such treated endothelial cells. However, the upregulation was less pronounced compared to the increase observed on HUVEC infected with HTNV (Figure 31).

Therefore, upregulation of antigen presentation molecules on the surface of hantavirus-infected HUVEC could not be solely attributed to viral properties. Some soluble factor secreted by the virus-infected cell might play a prominent role in inducing HLA class I-upregulation. IFN, however, are regularly involved in virus-

unspecific antiviral activity and, in consequence, they were strong candidates to start with. Moreover, the induction of IFN-β had already been demonstrated in HUVEC infected with both HTNV or TULV, even though to a different extent (see 3.4.3).

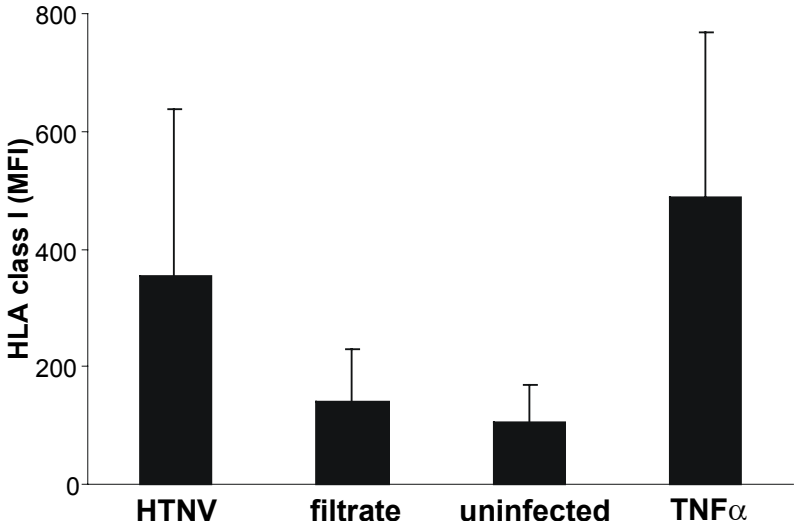

Figure 31. HLA class I expression after stimulation of HUVEC with filtrated virus-free supernatants of infected cells.

Cells were infected with HTNV (MOI 1). Supernatants of infected cells were collected three days after infection and virus was excluded by filtration. Untreated HUVEC were then stimulated with virus-free supernatants of infected cells for three days. Expression levels of HLA class I molecules were evaluated by flow cytometry analysis. Untreated HUVEC served as negative control. Direct infection with HTNV (MOI 1) and treatment with TNFα was used as positive controls. Cells were derived from three different donors and results are based upon three independent experiments and are given as mean ± SD.

Anti-IFN-β but not anti-IFN-α antibodies could inhibit HLA class I upregulation after hantavirus infection.

The contribution of IFN-β to the observed modulation of HLA class I molecules on HTNV- or TULV-infected HUVEC was investigated. For this purpose, neutralising anti-IFN-β antibodies or anti-IFN-α antibodies were used. In cultures with HTNV-infected HUVEC the presence of neutralising anti-IFN-β antibodies reduced upregulation of HLA class I molecules by 78 % (Figure 32). In contrast, neutralising anti-IFN-α antibodies had no effect (data not shown). In addition, anti-IFN-β antibodies were tested for their ability to interfere with TULV-induced HLA class I-upregulation. Despite the fact that TULV induced comparatively low amounts of IFN-β the HLA class I enhancement could be efficiently blocked by anti-IFN-β antibodies (81% inhibition). In contrast, anti-IFN-β antibodies did not prevent upregulation of

HLA class I molecules on uninfected control cells that had been treated with TNFα (Figure 32).

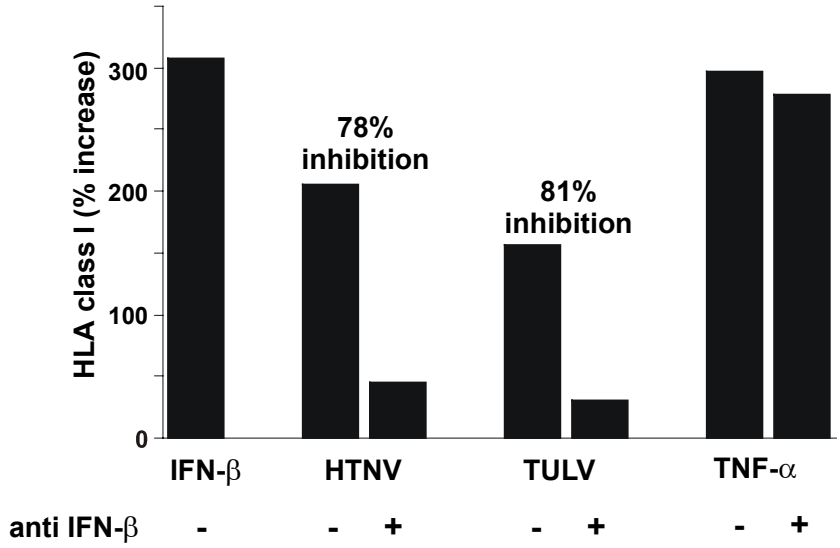

Figure 32. Inhibition of HLA class I-upregulation on HTNV-infected endothelial cells by anti-IFN-β antibodies.

HUVEC were infected with HTNV (MOI 1) and simultaneously treated with anti-IFN-α or anti-IFN-β antibodies. Uninfected cells were included as negative control. After three days of infection expression levels of HLA class I molecules were determined by flow cytometry analysis. Mean fluorescence intensity (MFI) values are given on the y-axis and are the mean ± SD derived from three individual experiments. In addition, the % inhibition of HLA class I-enhancement by anti-IFN-β antibodies was calculated and is shown above the righthand column.

In conclusion, these experiments indicate that IFN-β is for the most part responsible for the HTNV- and TULV-induced increase of HLA class I molecules on HUVEC. An important property of the innate immune system is, however, that it translates its encounter with viruses into an appropriate stimulation of the adaptive immune system. The limited role of direct effects in hantavirus-associated pathogenesis suggests that immune-mediated effector mechanisms may be involved. Indeed, several findings support the idea that hantaviruses can induce a vigorous cellular immune response. For example, in patients with acute HFRS, greatly increased numbers of activated CD8-positive T lymphocytes have been detected, resulting in a reversed CD4-positive/CD8-positive T lymphocyte ratio [Huang, 94]. Moreover, kidney biopsy specimens from patients with PUUV infection are characterised by infiltrating CD8-positive T lymphocytes [Temonen, 96]. In accordance with this description, hantavirus infection of endothelial cells has been

shown to trigger the expression of chemokines that attract T lymphocytes [Sundstrom, 01]. Activation of antiviral T lymphocytes, particularly in a primary response, depends critically on the proper function of monocyte-derived dendritic cells. Moreover, immature dendritic cells which reside in the dermis and epidermis of the skin and mucosa are among the first immune cells to encounter viruses after their entry into the human organism [Liu, 01]. These cells are also present in the epithelium and interstitium of the lungs, the portal of entry for hantaviruses. Therefore, the ability of pathogenic HTNV to infect human dendritic cells was investigated.

3.5 Infection of dendritic cells with hantaviruses.

For several reasons, DC represent a major link between the innate and adaptive immune responses. DC are the most potent antigen-presenting cells (APC) and the dominant cell type in the activation of naive T lymphocytes.

Immature or mature dendritic cells were infected with pathogenic HTNV at a very low MOI of 0.1. After four days of infection viral nucleocapsid protein could be detected in the cytoplasm of infected dendritic cells using immunohistochemistry. Up to 85 % of the immature and about 50 % of the mature dendritic cells stained positive at this time point. The punctuated structures and larger twisted threads mirrored the staining pattern observed in HTNV-infected HUVEC (Figure 33 A). Moreover, cytopathic effects were not observed in HTNV-infected dendritic cells by microscopic analysis at any time point during the course of infection.

To confirm productive infection viral titers in the supernatant of HTNV-infected immature dendritic cells were determined by focus test. The number of infectious particles released by both immature and mature dendritic cells increased during infection with HTNV (MOI of 0.05) until a peak was reached around day four postinfection (Figure 33 B). At later time points virus yields decreased and only low levels of secreted virus were detected eight days postinfection. Although the kinetic of infection was similar in both immature and mature dendritic cells, the total number of infectious particles produced by mature dendritic cells was much lower compared to immature dendritic cells.

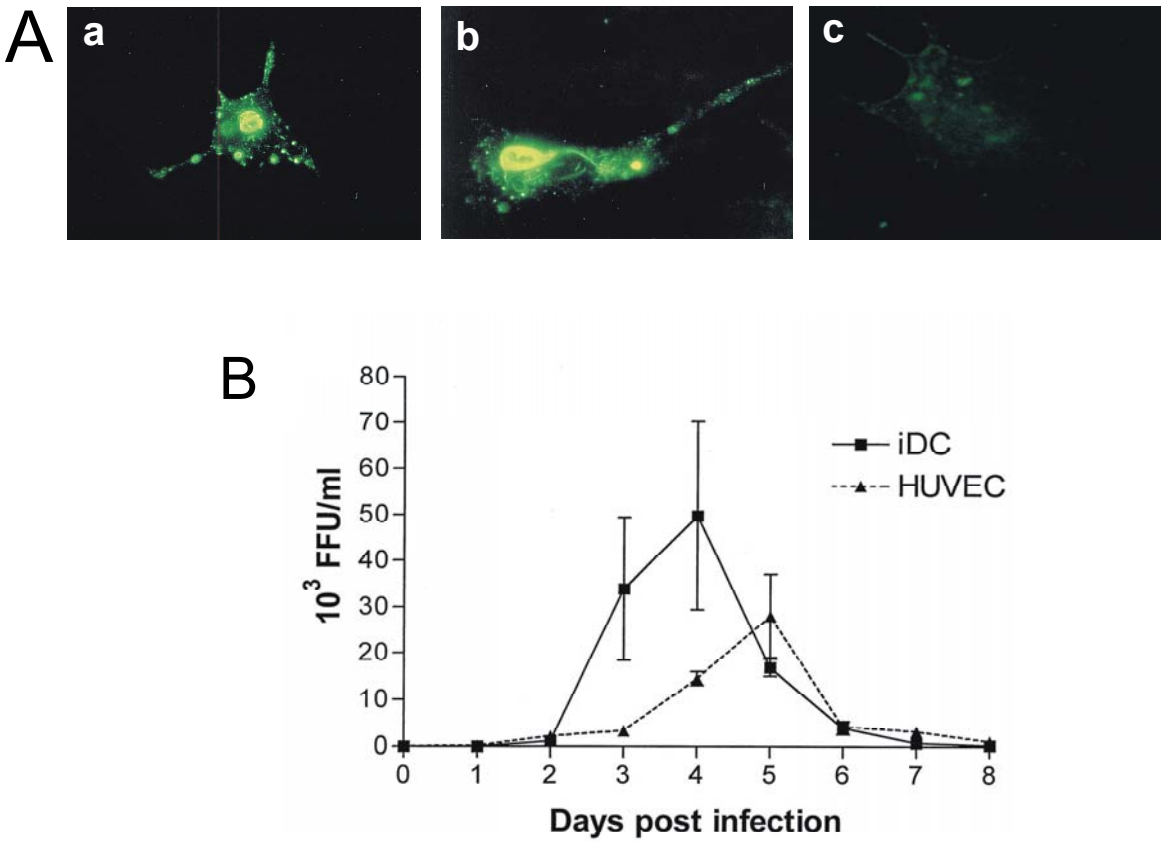

Figure 33. Detection of viral N protein and release of progeny virus in HTNV-infected dendritic cells.

(A) Cells were infected with HTNV (MOI 0.1) and analysed four days postinfection by immunohistochemistry. As a negative control, mock-infected immature dendritic cells were included in the analysis (c). The characteristic distribution of N protein was observed in monocyte-derived immature (a) and mature (b) dendritic cells. Original magnifications, ×63. (B) Monocyte-derived immature DC were inoculated with HTNV (MOI 0.05) for 1 h. Free virions were then removed by thoroughly washing the cells. At the time points indicated, supernatant was removed and centrifuged to remove cellular debris. Virus titer was then determined by focus assay.

Thus, both immature and mature dendritic cells were susceptible to an infection with pathogenic HTNV. In addition to this, dendritic cells support a productive infection, as could be demonstrated by the release of infectious particles into the supernatant [Raftery, 02].

4 Discussion

4.1 Safety aspects.

Laboratory transmission of hantaviruses from rodents to humans by aerosol exposure is well documented [Schmaljohn, 99]. Nonrodent-associated laboratory infections are rare, however, reports from laboratory-acquired infections with HTNV affirm the potential of a cell culture-adapted HTNV to infect humans [Van Epps, 99; Shi, 03]. It is therefore of crucial importance to investigate several different inactivation and clearance techniques and establish procedures for the complete inactivation or depletion of hantaviruses allowing the use of the treated samples in subsequent cell biological, virological and immunological assays. It is well established that enveloped viruses like hantaviruses are readily inactivated, as the destruction of the lipid membrane is accompanied by a total loss of infectivity. However, to leave the virus-infected cells almost unchanged and to preserve viral antigenic properties, heat inactivation or pH-mediated destruction as well as the use of harsh chemical treatment should be avoided.

4.1.1 Chemical inactivation procedures.

Methanol and acetone act as fat solvents and cause denaturation or coagulation of proteins. Therefore both solvents are efficient destroyers of enveloped viruses. In addition, fixation of cells with methanol has the dual effect of permeabilising them and at the same time allowing the dye to penetrate. Furthermore, the background fluorescence of the IFA sample is also reduced. Similarly, paraformaldehyde, like other aldehydes, causes denaturation of nucleic acids and proteins. Paraformaldehyde maintains cell structure and thereby allows staining of surface antigens making it a reasonable fixing agent for subsequent IFA and flow cytometry analyses. In line, it has been demonstrated that methanol, paraformaldehyde and acetone, used for the fixation of virus-infected cells, efficiently reduced the number of infectious particles below detection limits, i.e. by at least 6 to 7 log scales.

Recently, inactivation of West Nile virus in a detergent-containing buffer (0.05%

Tween 20) has been demonstrated [Mayo, 02]. In our studies performed with hantaviruses, a complete reduction of viral infectivity (at least 3 log scales) after lysing infected cells with Nonidet P-40™ containing lysis buffer could be verified. This procedure allows performance of Western blot analyses outside the biocontainment facilities.

4.1.2 Viral clearance by physical forces.

Another widely used method for virus inactivation is UV-irradiation [Freitas, 03; Thurston-Enriquez, 03]. UV-irradiation of HTNV for 3 minutes resulted in a reduction of infectivity of at least 6 log scales. However, the investigations revealed a clear dose-dependency for the inactivation kinetics as an incubation time of only one minute was not sufficient to clear the infectivity completely.

In contrast to the highly efficient virus inactivation by the procedures mentioned above, filtration using a 1,000 kDa pore size did not clear viral infectivity. A pore size of 300 kDa, however, reduced the viral infectivity to a level below limits of detection corresponding to a reduction of at least 5 log scales. This might give also some indication of the size of viral aggregates. Such filtration experiments suggest particle dimensions from 300 to 1,000 kDa which is in accordance with the calculated size of a single virion mass of about 420 kDa [Schmaljohn, 87].

4.1.3 Applicability of inactivated samples in further research assays.

The efficacy of the mentioned inactivation and removal procedures for the hantavirus type HTNV was verified during the present study. A reduction of infectivity by different chemical or physical inactivation and virus removal procedures was clearly demonstrated. In conclusion, different inactivation and depletion procedures for hantavirus and hantavirus-infected cells have been developed and evaluated. The efficient clearance of viral infectivity allows the use of HTNV or HTNV-infected cells in subsequent immunological, cell biological and virological assays. This is evidenced by the successful use of suchlike treated samples in subsequent immunofluorescence assays [Sibold, 01], Western blots (A.A. Kraus, unpubl. data), cell surface ELISAs (A.A. Kraus, unpubl. data) according to protocols described previously [Krüll, 96], and flow cytometry analyses [Raftery, 02].

4.2 The paradox of hantavirus-associated pathogenesis.

Autopsy findings of HFRS and HCPS victims typically reveal the common feature of increased permeability in microvascular beds, suggesting that the vascular endothelium may be a primary target for hantavirus infection. Further histological observations of tissue samples from people with HCPS who died demonstrated that hantaviruses do not cause detectable cytopathic effects in blood vessel endothelial cells, despite the presence of hantavirus antigens within these cells [Kanerva, 98; Zaki, 95]. This development of capillary leakage without visible endothelial damage is a major paradox of hantavirus pathogenesis. It once again points to the pivotal role the innate immune response might play in the course of a hantavirus infection. According to this, hemorrhagic fever viruses could modify hemostatic mechanisms in two separate ways:

The first is through direct action on cells involved in hemostasis, such as platelets and endothelial cells. This would comprise direct effects such as viral host-shutoff and could manifest itself in altered cell morphology, for instance. Increased permeability could be triggered through destruction of adherence-type junctions between endothelial cells or through degradation of the fibrin layer underneath that supports growth and viability of the endothelium. In fact, as former investigations have observed, vascular leakage can occur without endothelial cell damage or cell death [Franke, 87].

The second mechanism depends on the immune system. In the course of infection, immunopathological events could affect endothelial cells directly by triggering inflammatory pathways, including activation of transcription factors and MAP kinases, upregulation of adhesion molecules on the surface of infected cells, as well as induction of cytokines, namely of IFN. Furthermore, endothelial cells could also be subject to the destructive action of T lymphocytes after their clonal expansion due to the presence of infected dendritic cells.

These two pathogenic mechanisms, the direct one without the involvement of the immune system as well as the immunopathological one, could operate concurrently, with their relative importance dependent on the manifestations of disease, the infecting virus, and the person infected.

4.3 Direct viral effects on HUVEC after infection with hantaviruses.

Although hantaviruses are clearly infectious for endothelial cells, there is little evidence for direct injury caused by the viral infection. Our findings, that no general viral host-shutoff was detectable in infected cells correspond to findings of a former study [Pensiero, 92]. By using RT-PCR and Western blot analyses their work suggested that different endothelial cell gene products such IL-1, IL-6 or GAPDH were not affected in steady-state mRNA or protein levels. Similarly, in the present study levels of β-actin and cyclophilin B were not altered after infection with both HTNV or TULV as demonstrated in Western blot and RT-PCR analyses. In accordance with other studies [Hardestam, 03; Pensiero, 92; Zaki, 95], we did not observe morphological changes in hantavirus-infected endothelial cells. Dendritic cells could also be productively infected with HTNV *in vitro*, as demonstrated in the present study. HTNV did not change cell morphology in both cell types, though, and did not induce cell death by apoptotic signals mediated by viral replication in endothelial cells. However, HTNV delivered a strong maturation stimulus to immature dendritic cells as shown by phenotypic and functional changes and induced the release of proinflammatory cytokines [Raftery, 02].

Integrins have been identified as cellular receptors for hantaviruses [Mackow, 01]. These molecules, composed of α- and β-subunits, are involved in recognition of extracellular matrix proteins, cell-cell adhesion and platelet aggregation [Sugimori, 97]. Integrin subunits mediate cellular entry of hantaviruses and their pathogenic properties may depend on the usage of a particular integrin subtype. In fact, it has been shown previously, that pathogenic HTNV, SEOV and PUUV use $\alpha_V\beta_3$ integrins for cellular entry [Gavrilovskaya, 99]. Since β_3 integrins were recently reported to bind to receptors that regulate vascular permeability [Kevil, 98], it is conceivable that interactions between hantavirus and β_3 integrin may regulate endothelial cell function and contribute to viral pathogenesis.

Intriguingly, the $\alpha_v\beta_3$ integrin is one of the receptors involved in antigen uptake by phagocytes [Albert, 98]. Indeed, histopathological analysis of material from fatal HCPS cases could already reveal the presence of hantavirus-like particles not only in endothelial cells but also in dendritic cells, macrophages and lymphocytes [Zaki, 95].

4.3.1 Failure of hantaviruses to cause cytopathic effects in endothelial cells.

It could be demonstrated in the present study that primary human endothelial cells are highly susceptible to infection with both HTNV and TULV. However, *in vitro* infection with both HTNV or TULV did not lead to any detectable cytopathic effect as judged by light microscopy. These results are in line with former studies showing the capability of HTNV to infect Vero cells as well as human endothelial cells without causing noticeable cytopathic effects [Pensiero, 92]. Pathogenic events occuring after infection with hantaviruses in humans may therefore not be caused by direct viral damage to the cells but could be the consequence of immune mediated effects.

Productive infection of human endothelial cells, with or without cytopathology, has been demonstrated for many DNA and RNA viruses, including Dengue and Junin virus [Andrews, 78]. Both viruses can cause hemorrhagic fever in humans [Leduc, 89]. However, hemorrhagic fever viruses, including hantaviruses, do not necessarily cause cytopathic effects.

Generally, viral infection can lead to degenerative changes in infected cells on a biochemical or morphological basis. Such abnormalities often include shutdown of cellular RNA and protein synthesis and release of lysosomal enzymes. Visible morphological changes might comprise viral inclusion bodies, fusion with adjacent cells to form syncytia and diffuse changes in intracellular structures, and can be observed easily by light microscopy. Cytopathic effects should not be confused with necrosis. Necrosis describes the secondary changes that take place after cell death. Morphological features of a necrotic cell are much different from those of an infected cell, especially during the early stages of infection.

4.3.2 Apoptosis is not observed in cell culture after infection with hantaviruses.

Infection with hantaviruses neither changed cell morphology nor induced cell death in HUVEC or Vero E6 cells by apoptotic signals as judged by TUNEL assay. Yet another study could not observe effective apoptosis in Vero E6 cells after infection with HTNV, DOBV or PUUV at any time point either [Hardestam, 03]. In fact, there is still no clear-cut evidence for induction of apoptosis in infected Vero E6 cells. Admittedly, in a recent study, HTNV and PHV were shown to induce apoptosis in Vero E6 cells [Kang, 99]. However, apoptotic events were observed late after infection and at low levels only, with less than 30 percent of cells showing nuclear

DNA fragmentation seven days postinfection. And still on day 11 postinfection, about 40% of infected cells remained viable. It looks as if these unpaired findings do not indicate strong evidence for effective apoptosis in hantavirus-infected Vero E6 cells.

Another study again found that hantaviruses caused cytopathic effects and induced apoptosis in human embryonic kidney (HEK) cells [Markotic, 03]. It was observed, though, that apoptosis appeared mostly in uninfected adjacent cells and rarely in infected HEK cells. It is therefore conceivable that apoptosis occurred rather independently of hantavirus replication and production of some soluble factors contributed to apoptosis. The nature of such factor, however, was not identified. Since control experiments with inactivated hantaviruses were not performed and infection studies were conducted using varying application rates for different hantaviruses conclusions drawn from these experiments should be handled with care. Moreover, a variety of viruses like Herpes simplex virus, parainfluenza virus or respiratory syncytial virus have been found to cause cytopathic effects in HEK cells but not in other cell lines [Brown, 86].

Alternatively, hantaviruses might trigger apoptosis in infected cells in the first instance, but might then be able to counteract cell death. Virally induced expression of several anti-apoptotic proteins, for example, including cellular inhibitors of apoptosis (c-IAP) and cellular FLICE inhibitory protein (c-FLIP) could protect endothelial cells from cell death [Konopleva, 99]. However, induction of these anti-apoptotic molecules in hantavirus-infected endothelial cells remains to be elucidated in future studies.

4.4 Inflammatory events after infection with hantaviruses.

Inflammation is a protective response to infection by the immune system that requires communication between different classes of immune cells to coordinate their actions. It thereby involves different signalling pathways as well as various cellular signalling molecules:

The MAPK pathway, for instance, is stimulated by extracellular signals which initiate an intracellular cascade of phosphorylation events ultimately causing responses such as growth, differentiation, inflammation and apoptosis. Secreted cytokines, namely IFN, provide signals between immune cells to coordinate the inflammatory response. These cytokines, released by activated macrophages, for example, then trigger endothelial cells to increase expression levels of adhesion

molecules. Thereby, leukocytes are drawn to the site of inflammation by adherence. Finally, activation of NFκB is thought to be part of a stress response as it is activated by a variety of stimuli including growth factors, cytokines, and stress. This results in the translocation of the molecule to the nucleus, where it activates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, and inflammation, for instance.

4.4.1 Absence of NFκB activation or degradation of IκB-α after hantavirus infection.

Despite a broad variety of virus-induced pathways leading to the activation of NFκB, at the level of this central transcription factor and its inhibitor IκB-α cellular stress responses to infection with both HTNV or TULV could not be observed during this study. However, these experiments used an *in vitro* infection model and might not represent all *in vivo* events. It is possible that the mentioned proteins are indeed induced or signalling cascades are activated in human hosts. On the other hand, viral infection could change the activation state of molecules involved in signalling cascades. Inactivated phosphatases or inhibition of IκB kinases (IKK) due to viral manipulation could, for instance, prevent NFκB from disengaging from its inhibitor. Admittedly, the employed analytical techniques, mainly the IFA, are of limited sensitivity. Conclusions drawn from IFA are generally qualitative, rather than quantitative statements. It is therefore conceivable that minor levels of translocation of NFκB into the nucleus of an infected cell, have been overlooked. Using Western blot analysis, degradation of IκB to some extent might have been overlooked as well.

Activation of NFκB is a hallmark of most infections including viral infections. This transcription factor is normally found in the cytoplasm complexed with its inhibitory protein IκB. Upon infection, signalling events are initiated leading to phosphorylation of IκB by IKK-α or -β. Phosphorylated IκB is subsequently targeted for degradation through the ubiquitin-dependent proteasome pathway. Unmasked NFκB then migrates into the nucleus and activates transcription of NFκB-responsive genes. These genes encode, for example, a number of cytokines and their receptors, growth factors, cell adhesion molecules, and many other proteins. These proteins are involved in various processes, including immune responses, inflammation, cell growth and differentiation, as well as cell death by apoptosis. Most viruses studied to date activate NFκB, while a number of different mechanisms seems to be employed

[Mogensen, 01]. Viral envelope glycoproteins of herpesviruses, for instance, activate signalling through engagement of cellular receptors. Hepatitis B virus-encoded proteins, on the other hand, interact with intracellular signalling pathways and activate NFκB. Generally, accumulation of double-stranded RNA activates PKR, which in turn stimulates NFκB activity. Alternatively, overload of the endoplasmic reticulum (ER) due to massive adenovirus protein expression, for instance, can either lead to activation of NFκB.

4.4.2 MAP kinase signalling pathway is not activated after infection with HTNV.

During the studies presented here, phosphorylation of MAP kinases ERK and p38 was not detected in human endothelial cells at any time point postinfection, neither with HTNV nor with TULV.

These findings do not exclude the possibility that other p38-isoforms (p38-β, p38-γ or p38-δ) are activated. In fact, hints that these p38 homologes may not be regulated like p38 itself suggest that they define novel MAP kinase modules [Robinson, 97]. They might mediate type I IFN effects unrelated to regulation of gene transcription. Moreover, they might participate in the regulation of IFN-dependent gene transcription in a cell type-specific manner. Altogether, the precise regulatory mechanisms by which MAP kinases play a role in type I IFN-dependent transcription remain to be fully defined.

Finally, other types of endothelial cells, like microvascular endothelial cells (HMVEC) or lymphatic endothelial cells, might be more applicable endothelial cell types in the course of human hantavirus infection. It is conceivable that endothelial cells derived from different sources may react differently to hantavirus infection.

Generally, type I IFN transduce signals by activating the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to regulate transcription of target genes [Darnell, 94]. Additional signalling cascades are activated by type I IFN receptor, including the p38 MAP kinase pathway [David, 95]. Moreover, several MAP kinases phosphorylate STAT proteins allowing additional cellular signalling pathways to potentiate the primary STAT-activating stimulus [Decker, 00].

Among the different MAP kinase subgroups, i.e. ERK, p38 MAP kinases, and Jun-kinases (JNK), the p38 pathway may play a particular important role in IFN

signalling [Platanias, 03]. In a recent study, requirement for the p38 pathway in type I IFN-dependent gene transcription was indeed established. It could be demonstrated that the function of this MAP kinase cascade is essential for transcription of essentially all known type I IFN-dependent genes [Li, 04].

4.4.3 Weak upregulation of adhesion molecules after hantavirus infection.

Infection with HTNV or TULV did not lead to significantly increased expression levels of surface molecules such as ICAM-1, VCAM-1 or E-selectin, as could be demonstrated by using ELISA technique. Even though slight changes in the density of ICAM-1 molecules on the surface of infected endothelial cells were observed after six days of infection by the more sensitive flow cytometry technique, levels of modulation were altogether weak. A statistically significant increase could therefore not be determined. In accordance with these findings, a recent study could not detect significant virus-specific changes in the transcription levels of VCAM-1, ICAM-1, or E-selectin genes either [Sundstrom, 01]. However, a selective activation of chemokines RANTES and IP-10 was demonstrated and could provide a mechanism by which hantavirus infection may direct and perhaps enhance the effector immune response to the infected microvascular endothelium. It is conceivable that hantavirus infection of the endothelium may be necessary but insufficient to trigger increases in capillary leakage associated with HFRS. Both RANTES and IP-10 are predominantly chemotactic for mononuclear leukocytes, and IP-10 has been shown to be essential in the development of a protective T_H1 response against viral infections in the central nervous system [Liu, 00]. In contrast to these findings yet another study suggested that HTNV elicits a unique set of immune cell recruiting and attachment responses including VCAM, ICAM, and E-Selectin [Geimonen, 02]. These results were, however, not verified on the protein level but were solely based on the quantification of cellular mRNA levels.

4.5 Regulation of immunoregulatory proteins in hantavirus-infected HUVEC.

Type I IFN exert potent antiviral activities by inducing genes such as MxA and dsRNA-activated protein kinase that confer cellular resistance, inhibit viral replication, and block viral dissemination. They also serve important immunoregulatory roles by modulating expression of HLA molecules as well as adhesion molecules on the surface of infected cells.

4.5.1 Modulated expression levels of antigen presentation molecules.

A drastic increase in the density of HLA class I molecules after infection with both HTNV or TULV at MOI 1 or higher was observed during our studies. TULV-infected HUVEC appeared to upregulate HLA class I molecules even more rapidly. However, induction of HLA class I expression was not observed after infection with HTNV at MOI of 0.1 despite of the high sensitivity of the cytofluorimetric analysis compared to ELISA-based methods. Thus, modulation of immunoregulatory proteins on HUVEC was clearly dose-dependent. This may explain negative data from a previous study [Sundstrom, 01]. In fact, in that study upregulation of HLA class I molecules on the surface of hantavirus-infected HMVEC was not observed. This obvious discrepancy between the observed upregulation of HLA class I molecules on our hantavirus-infected primary HUVEC and the failure to do so in their primary HMVEC was dissolved during our kinetic studies using HTNV at considerably different MOI. This revealed the strong dose-dependent relationship for the observed HLA modulation. Neither HTNV nor TULV augmented the expression of HLA class II molecules on HUVEC as revealed by cytofluorimetric analyses.

Expression of HLA class I molecules is essential for the adaptive and innate antiviral immune response. These molecules serve as recognition elements for T cells and regulate natural killer (NK) cell function [Natarajan, 02]. Hence, different viruses decrease antigen processing and presentation which results in down-regulation of HLA molecules on infected cells, thus helping the viruses to escape the immune response [Alcami, 00; Tortorella, 00]. Only paramyxoviruses [Garofalo, 96], flaviviruses [King, 88], and coronaviruses [Suzumura, 86] have so far been reported to up-regulate the expression of HLA class I-molecules.

A possible mechanism underlying the phenomenon of rapid upregulation of HLA class I molecules on hantavirus-infected cells is the activation of NFκB. In fact, the promoter regions of HLA class I-genes contain NFκB binding sites. It has been reported, for example, that flavivirus-induced HLA class I upregulation is associated with NFκB translocation into the nucleus [Kesson, 01]. However, using already established techniques [Krüll, 96] we did not observe any changes in IκB levels and could not detect NFκB translocation into the nucleus.

In conclusion, hantavirus-induced upregulation of HLA class I-molecules might depend on mechanisms other than NFκB activation. Alternatively, expression of HLA class I molecules on the cell surface might be mainly regulated by the transporter

associated with antigen processing (TAP)-mediated import level of peptides into the ER rather than by the rate of biosynthesis of HLA class I molecules [Powis, 91]. Enhanced peptide import could then be due to augmented biosynthesis of TAP1 and TAP2. However, this hypothesis remains to be assessed in future studies.

4.5.2 Production of IFN in infected endothelial cells.

Many viruses enhance expression of HLA class I molecules indirectly through induction of type I IFN [Samuel, 01; Sen, 01]. In supernatants of HTNV-infected HUVEC we could detect only moderate levels of IFN-β. In comparison, even less IFN-β was found in supernatants of TULV-infected cells. Neither HTNV-infected nor TULV-infected cells released IFN-α. Given the fact that induction of IFN-α/β by viruses is primarily controlled at the transcriptional level quantitative real-time PCR was employed for a more detailed analysis. Peak levels of IFN-β mRNA were found in probes derived from HTNV-infected HUVEC at day 4 postinfection, while levels of IFN-β mRNA in TULV-infected HUVEC were altogether much lower. No induction of IFN-α, IFN-γ or IFN-λ was found by employing quantitative real-time RT-PCR. Similarly, in human endothelial cells from saphenous veins (HSVEC) increased IFN-β mRNA levels were found at day 3 but not at day 1 after infection with HTNV [Pensiero, 92]. HTNV-induced IFN-β mRNA production was also observed in yet another study using DNA array analyses at day 4 postinfection [Geimonen, 02]. However, PHV, a nonpathogenic hantavirus, failed to induce significant IFN-β mRNA levels in HUVEC in that study [Geimonen, 02]. The comparatively low levels of IFN-β mRNA in endothelial cells infected with nonpathogenic or rather non pathogenic hantaviruses could be due to inefficient viral replication and, hence, low production of double-stranded RNA which triggers synthesis of type I IFN.

One has to keep in mind, though, that related orthobunyaviruses possess an open reading frame in their S segments encoding for the IFN-antagonistic NSs protein [Kohl, 03; Weber, 02]. So far, an NSs protein has not been detected in hantavirus-infected cells. However, hantaviruses associated with rodents from subfamilies *Arvicolinae* and *Sigmodontinae* carry a second open reading frame (ORF-2) on the S segment, while those associated with *Murinae* rodents do not have such an ORF-2 [Plyusnin, 96]. Data from a very recent study suggest that the ORF-2 in both TULV and PUUV is functional and the encoded proteins can indeed antagonise the IFN response in infected cells [Tulimäki, 04]. This could be an

alternative explanation for the weak IFN response observed in cells infected with TULV. However, in the present study a genetic variant of TULV (strain Moravia) was used which carries a disrupted ORF-2 and does therefore not express a NSs protein (Rainer Ulrich, personal communication).

Activation of IFN gene expression is a cellular response resulting from innate immune recognition of virus infection. How cells sense viral replication, though, is only poorly characterised. While the generation of viral products such as double-stranded RNA plays a role in the activation of several cellular kinases, additional signals seem to stimulate phosphorylation of IRF-3 and IRF-7. These are essential events in induction of a protective IFN response [Smith, 01].

IFN signalling is not impaired in hantavirus-infected endothelial cells.

In our investigations enhanced levels of IFN-β mRNA were mirrored by increased expression levels of IRF-3 and –7. Moreover, an induction of transcription factor STAT-1 was observed in hantavirus-infected HUVEC. This is expected since both IRF-3 and IRF-7 are required to maximally induce the expression of type I IFN. STAT-1, however, is an important player in the type I IFN-triggered signalling pathway [Taniguchi, 01].

The virus-induced event that turns on IRF-3 includes activation of a serine/threonine kinase that phosphorylates IRF-3, allowing the protein to form dimers. These dimers then translocate to the nucleus and activate transcription. The kinase responsible for phosphorylation of IRF-3 has not been identified yet, but MAP kinase p38 has been suggested to be involved in the activation of IRF-3 by liposaccharide [Navarro, 99]. Activated IRF-3 then stimulates expression of IFN-β. Resulting IFN-β in turn stimulates production of IRF-7 which remains cytoplasmic in uninfected cells and becomes activated in virus-infected cells through a mechanism similar to that used by IRF-3 [Au, 98]. In addition, IRF-3 has been reported to stimulate Sendai virus-induced RANTES transcription in HEK cells [Lin, 99]. In fact, IRF-3 was also found to be upregulated in human endothelial cells after infection with hantaviruses [Sundstrom, 01].

Recent evidence for a type I IFN receptor (IFNAR)-, STAT-1-dependent signal occurring in the absence of ongoing immune responses to regulate HLA I expression suggests that a weak constitutive production of type I IFN keeps the host organism in a state of alertness to incoming virus [Taniguchi, 01]. Future studies will show if the

IRF-3/IRF-7 system is further involved in virus-induced expression of different cytokines. The field of virus-induced signalling, which may represent a general mechanism in host defence to virus infections, has at present been explored only minimally beyond the model viruses.

Hantavirus infection indirectly affects cellular mechanisms involved in hantavirus-induced pathogenesis.

The similar kinetics of HTNV-induced IFN-β production and HLA class I-expression suggested that this cytokine was responsible for the observed HLA class I-upregulation. By using neutralising antibodies we could show that IFN-β is indeed crucial for this phenotype. Anti-IFN-β but not anti-IFN-α antibodies could block up to 78 % of the virus-induced effect. Important human pathogens from several other RNA virus families also induce HLA class I expression through IFN-β on various cell types: It is known that human parainfluenza virus type 3 and respiratory syncytial virus increase levels of HLA class I molecules on respiratory epithelial cells via IFN-β production [Gao, 99; Garofalo, 96]. Similarly, IFN-β mediates induction of HLA class I expression on a glioma cell line and on HUVEC after infection with measles virus [Dhib-Jalbut, 93]. Moreover, HLA class I-expression induced by West Nile virus could be abrogated to a large extent by antibodies directed against type I IFN [Shen, 97].

Unexpectedly, HLA class I-enhancement associated with TULV-infection could also be prevented (up to 81%) with neutralising anti-IFN-β antibodies although TULV-infected cells produced only low amounts of IFN-β. It has been demonstrated that a weak IFN-β signal induced by autocrine/paracrine cytokine secretion is an essential component in a positive feedback loop [Taniguchi, 01] that could trigger a shortcut in the activation of the antiviral defense. In fact, induction of IFN-stimulated genes without enhanced synthesis of type I IFN has been described for virus infected cells [Navarro, 98] and could involve cellular receptors that recognise viral pathogen-associated molecular patterns (PAMPs) [Doyle, 02]. Thus, pathogenic hantaviruses like HTNV might be able to interfere with a weak IFN-β signal thereby disrupting the positive feed back loop.

4.6 Differential induction of IFN-dependent proteins and corresponding immune evasion.

The IFN system is one of the earliest defence mechanisms against virus infection aquired during evolution. Not surprisingly, millions of years of coevolution between hosts and their pathogens has resulted in the development of mechanisms by most viruses to evade, at least to some extent, the host IFN system. There are several hundred genes transcriptionally regulated by IFN [Der, 98]. Among them, three families of IFN-inducible genes have been extensively studied with respect to their antiviral activities. IFN-induced MxA, for instance, confers resistance to infection by several RNA viruses [Haller, 02]. Although the mechanism of action is not completely understood, experimental evidence supports a model in which MxA interferes with cellular trafficking of viral proteins. Generally, it appears that different viruses antagonise IFN-mediated defence responses by inhibiting distinct steps in IFN-activated signalling and antiviral pathways.

4.6.1 Different hantaviruses vary in their replication kinetics.

The results presented here demonstrate that human endothelial cells were highly susceptible to infection with both HTNV or TULV. However, although HUVEC were clearly capable of supporting growth of HTNV or TULV, production of infectious virus was limited to early time points after infection. This was then followed by the establishment of an antiviral state due to induction of IFN-β and MxA. Levels of virus production declined gradually over the next days and endothelial cells did not produce significant levels of infectious virus after a week of infection. Vero E6 cells, on the other hand, continued to produce high levels of virus for the duration of the experiments of 14 days and probably beyond [Pensiero, 92].

Generally, IFN-induced cytoplasmic MxA interferes with the replication of a variety of RNA viruses including hantaviruses, Rift valley fever virus or Crimean-Congo hemorrhagic fever virus [Andersson, 04; Frese, 95]. Vero E6 cells, however, are devoid of type I IFN genes because of a chromosomal deletion in their genome [Wathelet, 92]. Hence, they are unable to produce IFN-α/β upon viral infection and, as a consequence, do not express IFN-regulated genes, including those encoding endogenous MxA. Accordingly, these cells produce high viral titers after hantavirus

infection. Thus, endothelial cells can be productively infected with hantaviruses *in vitro* and they respond to the infection by activating antiviral signalling pathways.

The limited virus production in human endothelial cells may imply that the human vascular system is not an active reservoir for infectious virus during the course of disease. Indeed, recent reports suggested macrophages and dendritic cells as the target for virus spread in mice and humans [Raftery, 02].

Viral titers produced by TULV-infected human endothelial cells were much lower than in HTNV-infected endothelial cells, which generated peak titers at 48 hours postinfection. In contrast, on Vero E6 cells which are known to lack type I IFN genes [Wathelet, 92], TULV could grow as efficiently as HTNV. In fact, exogenously added human IFN all affect the replication of hantaviruses in Vero E6 cells [Tamura, 87].

Infected endothelial cells, which are not producing infectious virus still contain significant levels of viral antigen as detected by immunocytochemistry. Two plausible alternatives could explain this finding. First, hantavirus proteins may have an extremely long half-life. Secondly, IFN-β-mediated inhibition of hantavirus replication in endothelial cells occurs at some step in viral replication subsequent to translation of viral proteins. It has been demonstrated recently that the MxA protein sequesters the viral N protein thereby trapping this essential virus component in cytoplasmic inclusions. Hence, the nucleocapsid (N) protein becomes unavailable for the generation of new virus particles [Kochs, 02].

4.6.2 Early antiviral response is blocked by HTNV but not TULV.

By using Western blot analysis we could detect induction of MxA expression in HUVEC already 16 h after infection with TULV. This suggests that TULV allows weak IFN-β signalling which is involved in rapid induction of MxA expression. In this way TULV replication could be blocked at an early time point resulting in low virus titers. Accordingly, in Vero E6 cells, which are known to lack type I IFN genes and fail to mount an antiviral MxA response [Diaz, 88], TULV could grow as efficiently as HTNV. Supporting this view, in transfected Vero E6 cells expressing MxA replication of TULV is abrogated [Kanerva, 96a]. In contrast, HTNV delayed the MxA response in endothelial cells until 48 h postinfection. By this means pathogenic HTNV creates a time window from 16 h to 48 h postinfection in which it can replicate nearly as efficiently as in Vero E6 cells. Concomitant with the appearance of MxA the peak titer

was reached and subsequently declined. Taken together these data suggest that HTNV but not TULV is able to block the early antiviral immune response in human endothelial cells.

MxA proteins are IFN-induced GTPases that play a crucial role in the antiviral response against certain negative-strand RNA viruses [Haller, 98; Weber, 02]: *Orthomyxoviridae* [influenza A virus [Pavlovic, 90] and Thogoto virus [Frese, 95]], *Rhabdoviridae* [vesicular stomatitis virus [Pavlovic, 90]] and *Bunyaviridae* [La Crosse virus, Rift Valley fever virus, sandfly fever virus [Frese, 96], and HTNV [Frese, 96; Kanerva, 96a]]. It has been shown that MxA interferes with the transport of viral N protein to the Golgi compartment where assembly of hantaviruses takes place [Haller, 02]. Through this mechanism the N protein is no longer available for assembly of new virus particles and viral titers drop.

4.6.3 TULV is targeted by cellular antiviral activity, while HTNV is not – a case of viral immune evasion.

One of the evasion strategies that have been adopted by large DNA viruses is to encode homologues of cytokines, chemokines and their receptors, i.e. molecules that are of crucial importance for the control of the immune response. Human herpes virus 8, for instance, encodes four IRF homologues, which seem to inhibit cellular IRF. Alternatively, African swine fever virus blocks the NFκB pathway by gene products which mimick IκB, the cytoplasmic inhibitor of NFκB. By this means these viruses appear to block IFN production by directly inhibiting signal transduction pathways involved in IFN synthesis. It is widely thought, though, that viral double stranded (ds) RNA intermediates that accumulate during the course of replication are the primary mediators that trigger IFN production. dsRNA activates cellular kinases, including PKR, leading to the stimulation of several transcription factors which participate in promoting IFN expression, such as IRF or NFκB. However, dsRNA is not present when inactivated virus or a viral attachment protein is used.

Other viruses, including influenza viruses, encode dsRNA-binding proteins in order to inhibit activation of the dsRNA-induced pathway [Bergmann, 00]. Thus, IFN production is inhibited as well as the antiviral action of IFN in terms of preventing activation of dsRNA-induced PKR, which is transcriptionally induced by IFN.

However, viruses such as Ebola virus or Rift valley fever virus with comparatively small coding capacities had to develop different strategies to avoid the

host immune system. In fact, mechanisms used by these viruses to inhibit expression of type I IFN are still unknown [Levy, 01]. Likewise, strategies adopted by HTNV to evade the antiviral action of type I IFN are also not yet clarified.

The antiviral state is mediated through IFN-regulated cellular proteins such as MxA, OAS or PKR, which are synthesised after IFN exposure. All IFN-induced genes share a DNA element present in their 5' promoter regions, called the ISRE [Haque, 98]. To this sequence binds a nuclear factor, designated IFN-stimulated gene factor (ISGF), which promotes initiation of transcription and acts as enhancer [Ihle, 96]. The IFN signal transduction pathway is well known [Stark, 98]. Type I IFN receptors are heterodimers composed of two subunits. Appropriate ligand binding results in phosphorylation and activation of two tyrosine kinases associated with the subunits of the receptors, Jak1 and Tyk2. These proteins then phosphorylate STAT-1 and STAT-2. Phosphorylated STAT-1 and STAT-2 form a complex known as ISGF3 with p48, a DNA-binding protein. This complex translocates to the cell nucleus where it binds to ISRE sequences in the promoter region of IFN-stimulated genes, thus activating the transcriptional machinery. This pathway appears to be a target site for some viruses to escape host immune survey [Heim, 99; Miller, 99].

Since production of IFN in response to viral infection is one of the main defence mechanisms of the innate immune system, many viruses have developed strategies to subvert the IFN system. The S segment of Bunyamwera virus, the prototype of the *Bunyaviridae* family, for example, encodes two proteins, the nonstructural NSs protein and the N protein. While the NSs protein is not essential for viral replication and transcription, it has been shown that NSs inhibits induction of type I IFN [Weber, 02]. It acts as an IFN induction antagonist that blocks transcriptional activation of type I IFN in order to increase virulence of Bunyamwera virus. Moreover, as could be shown recently, Bunyamwera virus NSs protein can delay cell death in the early stages of infection by inhibiting IRF-3-mediated apoptosis [Kohl, 03]. Similarly, the NSs protein inherent to members of the Californian serogroup of bunyaviruses has also been shown to contribute to viral pathogenesis by inhibiting cellular transcription [Colon-Ramos, 03]. In contrast to other genera of *Bunyaviridae*, hantaviruses are not generally known to possess a sequence which would be similar to the ORF for the IFN-antagonistic NSs protein of related Orthobunyaviruses. In fact, only hantaviruses carried by *Arvicolinae* and *Sigmodontinae* rodents, but not those carried by *Murinae* rodents, have an ORF in the S segment. Both hantaviruses used during this study do

not encode an active NSs protein, though: HTNV like all other hantaviruses carried by *Murinae* rodents does not possess an ORF in the S segment. In general TULV, being associated with *Arvicolinae* rodents, carry an ORF in their S segment. The ORF of TULV strain Moravia, which was used during our studies, has a stop codon within this sequence and, hence, is not functional (Rainer Ulrich, personal communication).

It is, however, unclear by which means HTNV could interfere with the MxA response. It has been shown that the inducibility of the MxA protein, a recognised indicator of IFN action [von Wussow, 90], was inhibited totally in a cell line, which carried an integrated HBV genome. It was concluded that an HBV gene product might interact with some component of the IFN signalling pathway or at a pre-transcriptional level [Fernandez, 03]. Defective HBV genomes reduced antiviral activity of type I IFN mediated by the HBV core protein and this involved selective inhibition of the MxA protein but not other IFN-induced genes, such as PKR and OAS, as shown previously [Rosmorduc, 99]. In fact, these authors have proposed transcriptional inhibition of MxA promoter activity by the HBV core protein. In the study presented here, this antiviral protein, which is induced by type I IFN, might also be inhibited by HTNV. However, an inhibition of type I IFN on the transcriptional or translational level, without affecting other proteins, did not seem to be the reason, since a general protein-shutdown in infected cells was not observed. Moreover, it seemed, that IFN signalling was generally still functioning after an infection with HTNV, since an induction of IFN response genes, such as IRF-3, IRF-7, OAS or STAT was still observed by using RT-PCR and Western blot analyses. It is therefore conceivable that interference of HTNV with the MxA response occurred far downstream on the level of signalling, possibly by directly disrupting the MxA production.

TULV, on the other hand, only inefficiently induced the expression of IFN-β, probably due to a low replication rate. It was therefore a target of the antiviral activity of IFN-induced MxA protein. The comparatively high levels of HTNV, which imply high levels of viral protein production, point to a possible interaction of some viral proteins with the transcription machinery of the type I IFN system.

Of the innate immune response, type I IFN-induced factors appear to exert the most pronounced antiviral effect in hantavirus infection. The essential role of type I IFN in the host to combat infection is highlighted by the uncontrolled virus growth in

Vero E6 cells which are deficient in type I IFN genes. Moreover, type I IFN receptor knockout mice are even more susceptible to HTNV infections [Wichmann, 02]. There is evidence to suggest, however, that HTNV is very well able to interfere with IFN-induced host factors, namely with the MxA protein. This antivirally active protein was at least not induced by HTNV until two days of infection. In fact, strategies which confer resistance to type I IFN-induced host factors have been described for numerous other viruses, including hepatitis C virus and Vaccinia virus [Katze, 02].

4.7 Human hantavirus infection and the involvement of immune cells - Concept of immunopathogenesis.

HLA class I molecules, which serve as target structures for antiviral T cells, could be relevant to the hantavirus-associated pathogenesis as severity of the clinical course in humans may correlate with HLA type [Makela, 02; Mustonen, 96].

We could demonstrate for the first time that hantaviruses infect DC, which represent the most potent professional antigen-presenting cells (APC) of the host immune system. In an exhaustive study by Raftery et al. (2002) it could be demonstrated that hantaviruses, unlike HIV [Macatonia, 92], measles virus [Fugier-Vivier, 97; Grosjean, 97], or HCMV [Raftery, 01], induce upregulation of immunologically relevant molecules on DC. Expression of HLA class I and II molecules as well as the density of co-stimulatory molecules was increased representing a more mature phenotype. These phenotypic changes should be mirrored by functional changes. In fact, hantavirus-infected DC were able to stimulate T lymphocytes more efficiently, indicating an enhanced antigen presenting capacity of these important APC [Raftery, 02]. These findings imply that hantaviruses can elicit a strong T lymphocyte response which could significantly contribute to hantavirus-associated immunopathology.

Furthermore, it is conceivable that a delayed MxA response could allow pathogenic HTNV to spread more efficiently in the endothelial cell layer and upregulate HLA class I molecules on a higher proportion of cells (Figure 34). Accordingly, antiviral CD8-positive T lymphocytes may cause more extensive damage during elimination of cells infected with HTNV. Such effects could also be demonstrated for noncytolytic lymphocytic choriomeningitis virus (LCMV), for instance, as experiments with knockout mice could clearly show that tissue damage during infection requires CD8-positive T lymphocytes [Fung-Leung, 91]. In addition, increased IFN-β release by endothelial cells infected with pathogenic HTNV could

aggravate inflammatory processes that possibly contribute to endothelial dysfunction. Although precise functional consequences of these differences between pathogenic HTNV and rather nonpathogenic TULV remain to be elucidated *in vivo* they are most likely important for the pathogenesis of hantavirus-associated diseases.

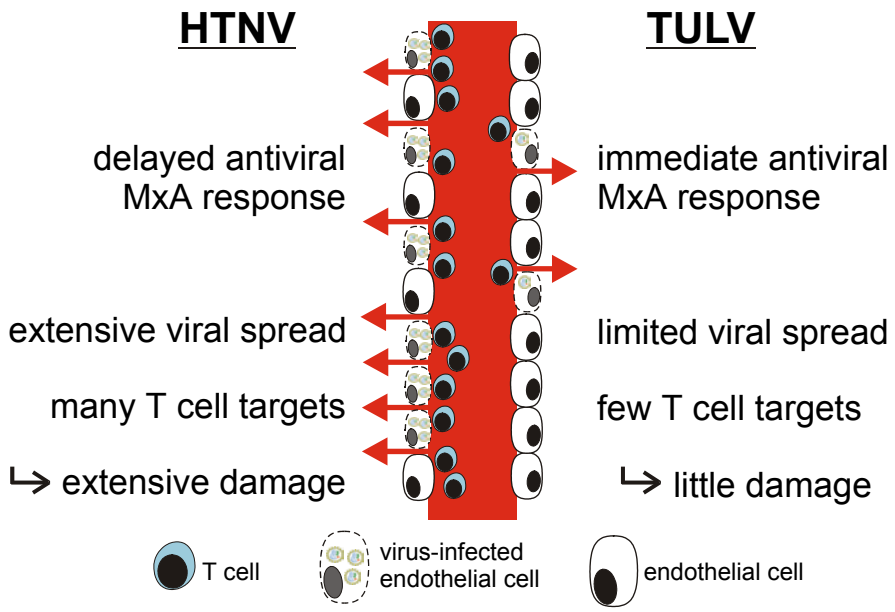

Figure 34. Differential antiviral response of endothelial cells.

Fast onset of the innate immune response, in particular an early induction of antivirally active MxA protein, limits viral spread. Therefore, only marginal damage is caused by TULV infection due to the comparatively low number of infected endothelial cells. In contrast, the MxA reponse is delayed after HTNV infection, which enables the virus to spread more efficiently in the endothelial cell layer. Consequently, extensive damage might be observed in the endothelial barrier since effector T lymphocytes have to destroy a relatively high number of endothelial cells to clear the viral infection.

Natural killer (NK) cell proliferation is induced by type I IFN, and the antiviral function of NK cells is thought to be important mainly in the first few days postinfection [Biron, 99]. It has been shown that flavivirus-infected target cells are significantly protected from lysis by *ex vivo* NK cells relative to uninfected targets [Lobigs, 03]. This might be a consquence of virus-mediated HLA class I upregulation [Lobigs, 96]. In case that kinetics of viremia coincides with that of the peak NK cell response observed in most viral infections *in vivo*, the viral effect on HLA class I expression might have physiological relevance in counteracting host NK cell defence. However, this hypothesis remains to be elucidated in detail for hantavirus infections.

In some viral hemorrhagic fevers, such as HFRS or Dengue hemorrhagic fever (DHF) as well as dengue shock syndrome (DSS), manifestations of disease appear to be clearly related to a powerful and destructive immune response [Lei, 01]. In

others, such as in Ebola virus infections, these manifestations seem to reflect compromised immunity with inability to check viremia and its consequences [Fisher-Hoch, 92]. In both cases, changes in cytokine elaboration could play a prominent role. For a human hantavirus infection, the picture could be like the following (Figure 35):

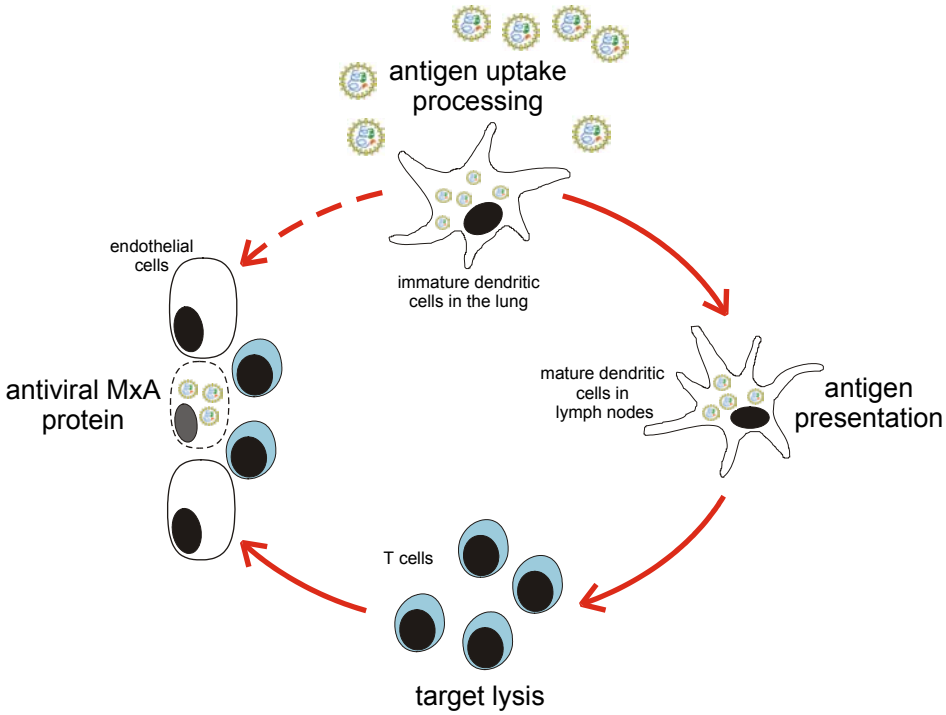

Figure 35. Hantaviruses and a concept of immunopathogenesis.

Immature dendritic cells in the lung are among the first cells to be infected by the virus. Hantavirus infection represents a strong maturation stimulus and infected dendritic cells aquire full T lymphocyte stimulatory capacity. Eventually, effector T lymphocytes reach the endothelium and eliminate endothelial cells expressing hantaviral antigens on their surface.

Endothelial cells and monocytes are thought to be the primary cell types infected by the virus, but infection has no direct cytopathic effect on these cells [Pensiero, 92; Temonen, 93; Zaki, 95]. Several studies have suggested that the clinical syndromes caused by hantavirus infection may be mediated in part by immunopathologic mechanisms, including the action of virus-specific CD8-positive and CD4-positive T lymphocytes [Van Epps, 99; Van Epps, 02]. Evidence to suggest this includes increases in the numbers of activated circulating CD8-positive T lymphocytes that are seen during HFRS and in kidney biopsies from patients with acute PUUV infections [Temonen, 96].

In airways and alveoli, in fact, a network of immature DC is located in the vicinity of epithelial cells and these important immune cells take up pathogens which are inhaled by humans. It has been shown that hantaviruses can productively infect

DC *in vitro* without causing significant cell death [Raftery, 02]. Infection of DC may facilitate transmission and dissemination of the virus throughout the body. In general, virus-infected DC are functionally impaired. However, DC infected with hantaviruses behave differently and acquire full T lymphocyte-stimulatory capacity *in vitro*. Therefore, it can be assumed that hantavirus-infected DC could efficiently stimulate a strong adaptive immune response against viral antigens in secondary lymphoid organs. Effector T lymphocytes resulting thereof could reach the infected organs via the bloodstream and eliminate endothelial cells expressing hantaviral antigens. In case of TULV this may cause only marginal damage because a fast onset of the innate immune response, i.e. expression of antiviral MxA protein, limits the number of infected endothelial cells. In contrast, we found that pathogenic HTNV delays the MxA-reponse allowing more efficient spread in the endothelial cell layer *in vitro* [Kraus, 04]. As a consequence effector T lymphocytes might have to destroy a relatively high number of endothelial cells to clear the viral infection thereby damaging the endothelial barrier and causing symptoms. In conclusion, peculiar interactions of hantaviruses with the host immune system might determine their pathogenicity.

The precise functional consequences of the differences between pathogenic and rather nonpathogenic hantaviruses remain to be elucidated *in vivo*. They are most likely of pivotal importance for the pathogenesis of hantavirus-associated diseases. Accordingly, it will be of great interest to see whether DC isolated from the animal hosts can also be infected by hantaviruses. To further test the *in vivo* relevance of hantavirus infection of DC the recently described syrian hamster model may provide a suitable means [Hooper, 01].

5 Reference List

Aaronson, D. S. und Horvath, C. M. (2002): A road map for those who know JAK-STAT, Science 296 [5573], Seite 1653-1655. URL: PM:12040185

Albert, M. L.; Pearce, S. F.; Francisco, L. M.; Sauter, B.; Roy, P.; Silverstein, R. L. und Bhardwaj, N. (1998): Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes, J.Exp.Med. 188 [7], Seite 1359-1368. URL: PM:9763615

Alcami, A. und Koszinowski, U. H. (2000): Viral mechanisms of immune evasion, Trends Microbiol. 8 [9], Seite 410-418. URL: PM:10989308

Andersson, I.; Bladh, L.; Mousavi-Jazi, M.; Magnusson, K. E.; Lundkvist, Å.; Haller, O. und Mirazimi, A. (2004): Human MxA protein inhibits the replication of Crimean-Congo hemorrhagic fever virus, J.Virol. 78 [8], Seite 4323-4329. URL: PM:15047845

Andrews, B. S.; Theofilopoulos, A. N.; Peters, C. J.; Loskutoff, D. J.; Brandt, W. E. und Dixon, F. J. (1978): Replication of dengue and junin viruses in cultured rabbit and human endothelial cells, Infect.Immun. 20 [3], Seite 776-781. URL: PM:208980

Arbabi, S. und Maier, R. V. (2002): Mitogen-activated protein kinases, Crit.Care Med. 30 [1 Suppl], Seite S74-S79. URL: PM:11782564

Au, W. C.; Moore, P. A.; LaFleur, D. W.; Tombal, B. und Pitha, P. M. (1998): Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes, J.Biol.Chem. 273 [44], Seite 29210-29217. URL: PM:9786932

Avsic-Zupanc, T.; Poljak, M.; Furlan, P.; Kaps, R.; Xiao, S. Y. und Leduc, J. W. (1994): Isolation of a strain of a Hantaan virus from a fatal case of hemorrhagic fever with renal syndrome in Slovenia, Am.J.Trop.Med.Hyg. 51 [4], Seite 393-400. URL: PM:7943563

Bergmann, M.; Garcia-Sastre, A.; Carnero, E.; Pehamberger, H.; Wolff, K.; Palese, P. und Muster, T. (2000): Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication, J.Virol. 74 [13], Seite 6203-6206. URL: PM:10846107

Bernshtein, A. D.; Apekina, N. S.; Mikhailova, T. V.; Myasnikov, Y. A.; Khlyap, L. A.; Korotkov, Y. S. und Gavrilovskaya, I. N. (1999): Dynamics of Puumala hantavirus infection in naturally infected bank voles (*Clethrionomys glareolus*), Arch.Virol. 144 [12], Seite 2415-2428. URL: PM:10664394

Bhardwaj, N. (1997): Interactions of viruses with dendritic cells: a double-edged sword, J.Exp.Med. 186 [6], Seite 795-799. URL: PM:9333647

Biron, C. A.; Nguyen, K. B.; Pien, G. C.; Cousens, L. P. und Salazar-Mather, T. P. (1999): Natural killer cells in antiviral defense: function and regulation by innate cytokines, Annu.Rev.Immunol. 17, Seite 189-220. URL: PM:10358757

Botten, J.; Mirowsky, K.; Kusewitt, D.; Bharadwaj, M.; Yee, J.; Ricci, R.; Feddersen, R. M. und Hjelle, B. (2000): Experimental infection model for Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*), Proc.Natl.Acad.Sci.U.S.A. 97 [19], Seite 10578-10583. URL: PM:10973478

Bowen, M. D.; Kariwa, H.; Rollin, P. E.; Peters, C. J. und Nichol, S. T. (1995): Genetic characterization of a human isolate of Puumala hantavirus from France, Virus Res. 38 [2-3], Seite 279-289. URL: PM:8578865

Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal.Biochem. 72, Seite 248-254. URL: PM:942051

Brown, M. und Petric, M. (1986): Evaluation of cell line 293 for virus isolation in routine viral diagnosis, J.Clin.Microbiol. 23 [4], Seite 704-708. URL: PM:3009540

CDC (1994): Hantavirus pulmonary syndrome - United States, 1993., MMWR Morb.Mortal.Wkly.Rep. 43 [RR-7], Seite 45-48.

CDC (1999): Biosafety in Microbiological and Biomedical Laboratories, 4th. Auflage , US Government Printing Office, Washington.

Chen, Z.; Hagler, J.; Palombella, V. J.; Melandri, F.; Scherer, D.; Ballard, D. und Maniatis, T. (1995): Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway, Genes Dev. 9 [13], Seite 1586-1597. URL: PM:7628694

Clark, E. A.; King, W. G.; Brugge, J. S.; Symons, M. und Hynes, R. O. (1998): Integrin-mediated signals regulated by members of the rho family of GTPases, J.Cell Biol. 142 [2], Seite 573-586. URL: PM:9679153

Clement, J. P. (2003): Hantavirus, Antiviral Res. 57 [1-2], Seite 121-127. URL: PM:12615308

Colon-Ramos, D. A.; Irusta, P. M.; Gan, E. C.; Olson, M. R.; Song, J.; Morimoto, R. I.; Elliott, R. M.; Lombard, M.; Hollingsworth, R.; Hardwick, J. M.; Smith, G. K. und Kornbluth, S. (2003): Inhibition of translation and induction of apoptosis by Bunyaviral nonstructural proteins bearing sequence similarity to reaper, Mol.Biol.Cell 14 [10], Seite 4162-4172. URL: PM:14517326

Cosgriff, T. M. (1991): Mechanisms of disease in Hantavirus infection: pathophysiology of hemorrhagic fever with renal syndrome, Rev.Infect.Dis. 13 [1], Seite 97-107. URL: PM:1673261

Darnell, J. E.; Kerr, I. M. und Stark, G. R. (1994): Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins, Science 264 [5164], Seite 1415-1421. URL: PM:8197455

David, M.; Petricoin, E.; Benjamin, C.; Pine, R.; Weber, M. J. und Larner, A. C. (1995): Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins, Science 269 [5231], Seite 1721-1723. URL: PM:7569900

De Caterina, R.; Bourcier, T.; Laufs, U.; La, Fata, V; Lazzerini, G.; Neish, A. S.; Libby, P. und Liao, J. K. (2001): Induction of endothelial-leukocyte interaction by interferon-gamma requires coactivation of nuclear factor-kappaB, Arterioscler.Thromb.Vasc.Biol. 21 [2], Seite 227-232. URL: PM:11156857

Decker, T. und Kovarik, P. (2000): Serine phosphorylation of STATs, Oncogene 19 [21], Seite 2628-2637. URL: PM:10851062

Der, S. D.; Zhou, A.; Williams, B. R. und Silverman, R. H. (1998): Identification of genes differentially regulated by interferon alpha, beta, or gamma using

oligonucleotide arrays, Proc.Natl.Acad.Sci.U.S.A. 95 [26], Seite 15623-15628. URL: PM:9861020

Dhib-Jalbut, S. S. und Cowan, E. P. (1993): Direct evidence that interferon-beta mediates enhanced HLA-class I expression in measles virus-infected cells, J.Immunol. 151 [11], Seite 6248-6258. URL: PM:8245464

Diaz, M. O.; Ziemin, S.; Le Beau, M. M.; Pitha, P.; Smith, S. D.; Chilcote, R. R. und Rowley, J. D. (1988): Homozygous deletion of the alpha- and beta 1-interferon genes in human leukemia and derived cell lines, Proc.Natl.Acad.Sci.U.S.A. 85 [14], Seite 5259-5263. URL: PM:3134658

Doyle, S.; Vaidya, S.; O'Connell, R.; Dadgostar, H.; Dempsey, P.; Wu, T.; Rao, G.; Sun, R.; Haberland, M.; Modlin, R. und Cheng, G. (2002): IRF3 mediates a TLR3/TLR4-specific antiviral gene program, Immunity. 17 [3], Seite 251-263. URL: PM:12354379

Farrell, H. E.; Vally, H.; Lynch, D. M.; Fleming, P.; Shellam, G. R.; Scalzo, A. A. und Davis-Poynter, N. J. (1997): Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo, Nature 386 [6624], Seite 510-514. URL: PM:9087412

Fernandez, M.; Quiroga, J. A. und Carreno, V. (2003): Hepatitis B virus downregulates the human interferon-inducible MxA promoter through direct interaction of precore/core proteins, J.Gen.Virol. 84 [Pt 8], Seite 2073-2082. URL: PM:12867637

Fisher-Hoch, S. P.; Brammer, T. L.; Trappier, S. G.; Hutwagner, L. C.; Farrar, B. B.; Ruo, S. L.; Brown, B. G.; Hermann, L. M.; Perez-Oronoz, G. I. und Goldsmith, C. S. (1992): Pathogenic potential of filoviruses: role of geographic origin of primate host and virus strain, J.Infect.Dis. 166 [4], Seite 753-763. URL: PM:1527410

Flohr, F.; Schneider-Schaulies, S.; Haller, O. und Kochs, G. (1999): The central interactive region of human MxA GTPase is involved in GTPase activation and interaction with viral target structures, FEBS Lett. 463 [1-2], Seite 24-28. URL: PM:10601631

Franke, W. W.; Kapprell, H. P. und Cowin, P. (1987): Immunolocalization of plakoglobin in endothelial junctions: identification as a special type of Zonulae adhaerentes, Biol.Cell 59 [3], Seite 205-218. URL: PM:2955839

Freitas, T. R.; Gaspar, L. P.; Caldas, L. A.; Silva, J. L. und Rebello, M. A. (2003): Inactivation of classical swine fever virus: association of hydrostatic pressure and ultraviolet irradiation, J.Virol.Methods 108 [2], Seite 205-211. URL: PM:12609688

French, G. R.; Foulke, R. S.; Brand, O. A.; Eddy, G. A.; Lee, H. W. und Lee, P. W. (1981): Korean hemorrhagic fever: propagation of the etiologic agent in a cell line of human origin, Science 211 [4486], Seite 1046-1048. URL: PM:6110243

Frese, M.; Kochs, G.; Feldmann, H.; Hertkorn, C. und Haller, O. (1996): Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein, J.Virol. 70 [2], Seite 915-923. URL: PM:8551631

Frese, M.; Kochs, G.; Meier-Dieter, U.; Siebler, J. und Haller, O. (1995): Human MxA protein inhibits tick-borne Thogoto virus but not Dhori virus, J.Virol. 69 [6], Seite 3904-3909. URL: PM:7745744

Fugier-Vivier, I.; Servet-Delprat, C.; Rivailler, P.; Rissoan, M. C.; Liu, Y. J. und Rabourdin-Combe, C. (1997): Measles virus suppresses cell-mediated immunity by

interfering with the survival and functions of dendritic and T cells, J.Exp.Med. 186 [6], Seite 813-823. URL: PM:9294136

Fung-Leung, W. P.; Kundig, T. M.; Zinkernagel, R. M. und Mak, T. W. (1991): Immune response against lymphocytic choriomeningitis virus infection in mice without CD8 expression, J.Exp.Med. 174 [6], Seite 1425-1429. URL: PM:1683893

Galeno, H.; Mora, J.; Villagra, E.; Fernandez, J.; Hernandez, J.; Mertz, G. J. und Ramirez, E. (2002): First human isolate of hantavirus (Andes virus) in the Americas, Emerg.Infect.Dis. 8 [7], Seite 657-661. URL: PM:12095430

Gao, J.; De, B. P. und Banerjee, A. K. (1999): Human parainfluenza virus type 3 up-regulates major histocompatibility complex class I and II expression on respiratory epithelial cells., J.Virol. 73 [2], Seite 1411-1418. URL: PM:9882346

Garofalo, R.; Mei, F.; Espejo, R.; Ye, G.; Haeberle, H.; Baron, S.; Ogra, P. L. und Reyes, V. E. (1996): Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class I MHC expression through the induction of IFN-beta and IL-1 alpha, J.Immunol. 157 [6], Seite 2506-2513. URL: PM:8805651

Gavrieli, Y.; Sherman, Y. und Ben Sasson, S. A. (1992): Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J.Cell Biol. 119 [3], Seite 493-501. URL: PM:1400587

Gavrilovskaya, I. N.; Brown, E. J.; Ginsberg, M. H. und Mackow, E. R. (1999): Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by beta3 integrins, J.Virol. 73 [5], Seite 3951-3959. URL: PM:10196290

Gavrilovskaya, I. N.; Peresleni, T.; Geimonen, E. und Mackow, E. R. (2002): Pathogenic hantaviruses selectively inhibit beta3 integrin directed endothelial cell migration, Arch.Virol. 147 [10], Seite 1913-1931. URL: PM:12376753

Gavrilovskaya, I. N.; Shepley, M.; Shaw, R.; Ginsberg, M. H. und Mackow, E. R. (1998): beta3 integrins mediate the cellular entry of hantaviruses that cause respiratory failure, Proc.Natl.Acad.Sci.U.S.A. 95 [12], Seite 7074-7079. URL: PM:9618541

Geimonen, E.; Neff, S.; Raymond, T.; Kocer, S. S.; Gavrilovskaya, I. N. und Mackow, E. R. (2002): Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses, Proc.Natl.Acad.Sci.U.S.A. 99 [21], Seite 13837-13842. URL: PM:12368479

Gonzalez, J. P.; McCormick, J. B.; Baudon, D.; Gautun, J. P.; Meunier, D. Y.; Dournon, E. und Georges, A. J. (1984): Serological evidence for Hantaan-related virus in Africa, Lancet 2 [8410], Seite 1036-1037. URL: PM:6149414

Gonzalez-Scarano, F. und Nathanson, N. (1996): *Bunyaviridae*, Fields Virology, B.N.Fields,D.M.Knipe & P.M.Howley (eds.) , 3rd. Auflage, Seite 1473-1504, Lippincott - Raven, Philadelphia, ISBN: 0-7817-0253-4.

Gratton, J. P.; Morales-Ruiz, M.; Kureishi, Y.; Fulton, D.; Walsh, K. und Sessa, W. C. (2001): Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells, J.Biol.Chem. 276 [32], Seite 30359-30365. URL: PM:11387313

Green, W.; Feddersen, R.; Yousef, O.; Behr, M.; Smith, K.; Nestler, J.; Jenison, S.; Yamada, T. und Hjelle, B. (1998): Tissue distribution of hantavirus antigen in naturally infected humans and deer mice, J.Infect.Dis. 177 [6], Seite 1696-1700. URL: PM:9607851

Groen, J.; Dalrymple, J.; Fisher-Hoch, S.; Jordans, J. G.; Clement, J. P. und Osterhaus, A. D. (1992): Serum antibodies to structural proteins of hantavirus arise at different times after infection, J.Med.Virol. 37 [4], Seite 283-287. URL: PM:1357082

Grosjean, I.; Caux, C.; Bella, C.; Berger, I.; Wild, F.; Banchereau, J. und Kaiserlian, D. (1997): Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells, J.Exp.Med. 186 [6], Seite 801-812. URL: PM:9294135

Gui, X. E.; Ho, M.; Cohen, M. S.; Wang, Q. L.; Huang, H. P. und Xie, Q. X. (1987): Hemorrhagic fever with renal syndrome: treatment with recombinant alpha interferon, J.Infect.Dis. 155 [5], Seite 1047-1051. URL: PM:2881968

Guidotti, L. G. und Chisari, F. V. (2001): Noncytolytic control of viral infections by the innate and adaptive immune response, Annu.Rev.Immunol. 19, Seite 65-91. URL: PM:11244031

Hack, C. E. und Zeerleder, S. (2001): The endothelium in sepsis: source of and a target for inflammation, Crit.Care Med. 29 [7 Suppl], Seite S21-S27. URL: PM:11445730

Haller, O.; Frese, M. und Kochs, G. (1998): Mx proteins: mediators of innate resistance to RNA viruses, Rev.Sci.Tech. 17 [1], Seite 220-230. URL: PM:9638812

Haller, O. und Kochs, G. (2002): Interferon-induced Mx proteins: dynamin-like GTPases with antiviral activity, Traffic. 3 [10], Seite 710-717. URL: PM:12230469

Haque, S. J. und Williams, B. R. (1998): Signal transduction in the interferon system, Semin.Oncol. 25 [1 Suppl 1], Seite 14-22. URL: PM:9482536

Hardestam, J.; Klingström, J. und Lundkvist, Å. (2003): Apoptosis is not induced in Vero E6 cells after hantavirus infection., Abstracts XII International conference on negative strand viruses, Pisa., Seite 85.

Hart, C. A. und Bennett, M. (1999): Hantavirus infections: epidemiology and pathogenesis, Microbes.Infect. 1 [14], Seite 1229-1237. URL: PM:10580279

Heider, H.; Ziaja, B.; Priemer, C.; Lundkvist, Å.; Neyts, J.; Krüger, D. H. und Ulrich, R. (2001): A chemiluminescence detection method of hantaviral antigens in neutralisation assays and inhibitor studies, J.Virol.Methods 96 [1], Seite 17-23. URL: PM:11516485

Heim, M. H.; Moradpour, D. und Blum, H. E. (1999): Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway, J.Virol. 73 [10], Seite 8469-8475. URL: PM:10482599

Hiscott, J.; Kwon, H. und Genin, P. (2001): Hostile takeovers: viral appropriation of the NF-kappaB pathway, J.Clin.Invest 107 [2], Seite 143-151. URL: PM:11160127

Hiscott, J.; Nguyen, H. und R.Lin (1995): Molecular mechanisms of interferon beta gene induction, Seminars in Virology 6, Seite 161-173.

Hiscott, J.; Pitha, P.; Genin, P.; Nguyen, H.; Heylbroeck, C.; Mamane, Y.; Algarte, M. und Lin, R. (1999): Triggering the interferon response: the role of IRF-3 transcription factor, J.Interferon Cytokine Res. 19 [1], Seite 1-13. URL: PM:10048763

Hjelle, B.; Goade, D.; Torrez-Martinez, N.; Lang-Williams, M.; Kim, J.; Harris, R. L. und Rawlings, J. A. (1996): Hantavirus pulmonary syndrome, renal insufficiency, and myositis associated with infection by Bayou hantavirus, Clin.Infect.Dis. 23 [3], Seite 495-500. URL: PM:8879771

Hooper, J. W.; Larsen, T.; Custer, D. M. und Schmaljohn, C. S. (2001): A lethal disease model for hantavirus pulmonary syndrome, Virology 289 [1], Seite 6-14. URL: PM:11601912

Huang, C.; Jin, B.; Wang, M.; Li, E. und Sun, C. (1994): Hemorrhagic fever with renal syndrome: relationship between pathogenesis and cellular immunity, J.Infect.Dis. 169 [4], Seite 868-870. URL: PM:7510765

Huppertz, B.; Frank, H. G. und Kaufmann, P. (1999): The apoptosis cascade--morphological and immunohistochemical methods for its visualization, Anat.Embryol.(Berlin) 200 [1], Seite 1-18. URL: PM:10395001

Hynes, R. O. (1992a): Integrins: versatility, modulation, and signaling in cell adhesion, Cell 69 [1], Seite 11-25. URL: PM:1555235

Hynes, R. O. und Lander, A. D. (1992b): Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons, Cell 68 [2], Seite 303-322. URL: PM:1733501

Ihle, J. N. (1996): STATs: signal transducers and activators of transcription, Cell 84 [3], Seite 331-334. URL: PM:8608586

Iordanov, M. S.; Paranjape, J. M.; Zhou, A.; Wong, J.; Williams, B. R.; Meurs, E. F.; Silverman, R. H. und Magun, B. E. (2000): Activation of p38 mitogen-activated protein kinase and c-Jun NH_2-terminal kinase by double-stranded RNA and encephalomyocarditis virus: involvement of RNase L, protein kinase R, and alternative pathways, Mol.Cell Biol. 20 [2], Seite 617-627. URL: PM:10611240

Jaffe, E. A.; Nachman, R. L.; Becker, C. G. und Minick, C. R. (1973): Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria, J.Clin.Invest 52 [11], Seite 2745-2756. URL: PM:4355998

Johnson-Leger, C. und Imhof, B. A. (2003): Forging the endothelium during inflammation: pushing at a half-open door?, Cell Tissue Res. 314, Seite 93-105. URL: PM:12955495

Jonsson, C. B. und Schmaljohn, C. S. (2001): Replication of hantaviruses, Curr.Top.Microbiol.Immunol. 256, Seite 15-32. URL: PM:11217402

Juto, P.; Elgh, F.; Ahlm, C.; Alexeyev, O. A.; Edlund, K.; Lundkvist, Å. und Wadell, G. (1997): The first human isolate of Puumala virus in Scandinavia as cultured from phytohemagglutinin stimulated leucocytes, J.Med.Virol. 53 [2], Seite 150-156. URL: PM:9334926

Kanerva, M.; Melen, K.; Vaheri, A. und Julkunen, I. (1996a): Inhibition of Puumala and Tula hantaviruses in Vero cells by MxA protein, Virology 224 [1], Seite 55-62. URL: PM:8862399

Kanerva, M.; Mustonen, J. und Vaheri, A. (1998): Pathogenesis of Puumala and other hantavirus infections, Rev.Med.Virol. 8 [2], Seite 67-86. URL: PM:10398496

Kanerva, M.; Paakkala, A.; Mustonen, J.; Paakkala, T.; Lahtela, J. und Pasternack, A. (1996b): Pulmonary involvement in nephropathia epidemica: radiological findings and their clinical correlations, Clin.Nephrol. 46 [6], Seite 369-378. URL: PM:8982552

Kang, J. I.; Park, S. H.; Lee, P. W. und Ahn, B. Y. (1999): Apoptosis is induced by hantaviruses in cultured cells, Virology 264 [1], Seite 99-105. URL: PM:10544134

Katze, M. G.; He, Y. und Gale, M., Jr. (2002): Viruses and interferon: a fight for supremacy, Nat.Rev.Immunol. 2 [9], Seite 675-687. URL: PM:12209136

Kesson, A. M. und King, N. J. (2001): Transcriptional regulation of major histocompatibility complex class I by flavivirus West Nile is dependent on NF-kappaB activation, J.Infect.Dis. 184 [8], Seite 947-954. URL: PM:11574908

Kevil, C. G.; Payne, D. K.; Mire, E. und Alexander, J. S. (1998): Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins, J.Biol.Chem. 273 [24], Seite 15099-15103. URL: PM:9614120

Khaiboullina, S. F. und St.Jeor, S. C. (2002): Hantavirus immunology, Viral Immunol. 15 [4], Seite 609-625. URL: PM:12513931

Kim, G. R. und McKee, K. T., Jr. (1985): Pathogenesis of Hantaan virus infection in suckling mice: clinical, virologic, and serologic observations, Am.J.Trop.Med.Hyg. 34 [2], Seite 388-395. URL: PM:2858987

King, N. J. und Kesson, A. M. (1988): Interferon-independent increases in class I major histocompatibility complex antigen expression follow flavivirus infection, J.Gen.Virol. 69, Seite 2535-2543. URL: PM:2844965

Kitamura, T.; Morita, C.; Komatsu, T.; Sugiyama, K.; Arikawa, J.; Shiga, S.; Takeda, H.; Akao, Y.; Imaizumi, K.; Oya, A.; Hashimoto, N. und Urasawa, S. (1983): Isolation of virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system, Jpn.J.Med.Sci.Biol. 36 [1], Seite 17-25. URL: PM:6134854

Klempa, B.; Meisel, H.; Rath, S.; Bartel, J.; Ulrich, R. und Krüger, D. H. (2003): Occurrence of renal and pulmonary syndrome in a region of northeast Germany where Tula hantavirus circulates, J.Clin.Microbiol. 41 [10], Seite 4894-4897. URL: PM:14532254

Klingström, J.; Plyusnin, A.; Vaheri, A. und Lundkvist, Å. (2002): Wild-type Puumala hantavirus infection induces cytokines, C-reactive protein, creatinine, and nitric oxide in cynomolgus macaques, J.Virol. 76 [1], Seite 444-449. URL: PM:11739712

Kluck, R. M.; Bossy-Wetzel, E.; Green, D. R. und Newmeyer, D. D. (1997): The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis, Science 275 [5303], Seite 1132-1136. URL: PM:9027315

Kochs, G.; Janzen, C.; Hohenberg, H. und Haller, O. (2002): Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes, Proc.Natl.Acad.Sci.U.S.A. 99 [5], Seite 3153-3158. URL: PM:11880649

Kohl, A.; Clayton, R. F.; Weber, F.; Bridgen, A.; Randall, R. E. und Elliott, R. M. (2003): Bunyamwera virus nonstructural protein NSs counteracts interferon regulatory factor 3-mediated induction of early cell death, J.Virol. 77 [14], Seite 7999-8008. URL: PM:12829839

Konopleva, M.; Zhao, S.; Xie, Z.; Segall, H.; Younes, A.; Claxton, D. F.; Estrov, Z.; Kornblau, S. M. und Andreeff, M. (1999): Apoptosis. Molecules and mechanisms, Adv.Exp.Med.Biol. 457, Seite 217-236. URL: PM:10500797

Korsmeyer, S. J. (1999): BCL-2 gene family and the regulation of programmed cell death, Cancer Res. 59 [7 Suppl], Seite 1693s-1700s. URL: PM:10197582

Krakauer, T.; Leduc, J. W.; Morrill, J. C.; Anderson, A. O. und Krakauer, H. (1994): Serum levels of alpha and gamma interferons in hemorrhagic fever with renal syndrome, Viral Immunol. 7 [2], Seite 97-101. URL: PM:7848512

Kraus, A. A.; Raftery, M. J.; Giese, T.; Ulrich, R.; Zawatzky, R.; Hippenstiel, S.; Suttorp, N.; Krüger, D. H. und Schönrich, G. (2004): Differential antiviral response of endothelial cells after infection with pathogenic and nonpathogenic hantaviruses, J.Virol. 78 [12], Seite 6143-6150. URL: PM:15163707

Krüger, D. H.; Ulrich, R. und Lundkvist, Å. (2001): Hantavirus infections and their prevention, Microbes.Infect. 3 [13], Seite 1129-1144. URL: PM:11709294

Krüll, M.; Dold, C.; Hippenstiel, S.; Rosseau, S.; Lohmeyer, J. und Suttorp, N. (1996): *Escherichia coli* hemolysin and *Staphylococcus aureas* alpha-toxin potently induce neutrophil adhesion to cultured human endothelial cells, J.Immunol. 157 [9], Seite 4133-4140. URL: PM:8892649

Ksiazek, T. G.; Peters, C. J.; Rollin, P. E.; Zaki, S.; Nichol, S.; Spiropoulou, C.; Morzunov, S.; Feldmann, H.; Sanchez, A. und Khan, A. S. (1995): Identification of a new North American hantavirus that causes acute pulmonary insufficiency, Am.J.Trop.Med.Hyg. 52 [2], Seite 117-123. URL: PM:7872437

Kuhns, D. B.; Alvord, W. G. und Gallin, J. I. (1995): Increased circulating cytokines, cytokine antagonists, and E-selectin after intravenous administration of endotoxin in humans, J.Infect.Dis. 171 [1], Seite 145-152. URL: PM:7528250

Lampugnani, M. G.; Resnati, M.; Dejana, E. und Marchisio, P. C. (1991): The role of integrins in the maintenance of endothelial monolayer integrity, J.Cell Biol. 112 [3], Seite 479-490. URL: PM:1899416

Leduc, J. W. (1989): Epidemiology of hemorrhagic fever viruses, Rev.Infect.Dis. 11 Suppl 4, Seite S730-S735. URL: PM:2546247

Lee, H. W. (1996): Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome, The *Bunyaviridae*, Elliott R.M. (ed.) , Seite 253-264, Plenum Press, New York.

Lee, H. W.; Lee, P. W. und Johnson, K. M. (1978): Isolation of the etiologic agent of Korean Hemorrhagic fever, J.Infect.Dis. 137 [3], Seite 298-308. URL: PM:24670

Lee, H. W. und van der Groen, G. (1989): Hemorrhagic fever with renal syndrome, Prog.Med.Virol. 36, Seite 62-102. URL: PM:2573914

Legendre, F.; Dudhia, J.; Pujol, J. P. und Bogdanowicz, P. (2003): JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of Type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of SOX9 expression, J.Biol.Chem. 278 [5], Seite 2903-2912. URL: PM:12419823

Lei, H. Y.; Yeh, T. M.; Liu, H. S.; Lin, Y. S.; Chen, S. H. und Liu, C. C. (2001): Immunopathogenesis of dengue virus infection, J.Biomed.Sci. 8 [5], Seite 377-388. URL: PM:11549879

Levy, D. E. und Garcia-Sastre, A. (2001): The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion, Cytokine Growth Factor Rev. 12 [2-3], Seite 143-156. URL: PM:11325598

Lewis, R. M.; Lee, H. W.; See, A. F.; Parrish, D. B.; Moon, J. S.; Kim, D. J. und Cosgriff, T. M. (1991): Changes in populations of immune effector cells during the course of hemorrhagic fever with renal syndrome, Trans.R.Soc.Trop.Med.Hyg. 85 [2], Seite 282-286. URL: PM:1679572

Li, Y.; Sassano, A.; Majchrzak, B.; Deb, D. K.; Levy, D. E.; Gaestel, M.; Nebreda, A. R.; Fish, E. N. und Platanias, L. C. (2004): Role of p38α Map kinase in type I interferon signaling, J.Biol.Chem. 279 [2], Seite 970-979. URL: PM:14578350

Lin, R.; Heylbroeck, C.; Genin, P.; Pitha, P. M. und Hiscott, J. (1999): Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription, Mol.Cell Biol. 19 [2], Seite 959-966. URL: PM:9891032

Linderholm, M.; Bjermer, L.; Juto, P.; Roos, G.; Sandstrom, T.; Settergren, B. und Tarnvik, A. (1993): Local host response in the lower respiratory tract in nephropathia epidemica, Scand.J.Infect.Dis. 25 [5], Seite 639-646. URL: PM:7904380

Liu, M. T.; Chen, B. P.; Oertel, P.; Buchmeier, M. J.; Armstrong, D.; Hamilton, T. A. und Lane, T. E. (2000): The T cell chemoattractant IFN-inducible protein 10 is essential in host defense against viral-induced neurologic disease, J.Immunol. 165 [5], Seite 2327-2330. URL: PM:10946253

Liu, Y. J. (2001): Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity, Cell 106 [3], Seite 259-262. URL: PM:11509173

Ljunggren, H. G. und Karre, K. (1990): In search of the 'missing self': MHC molecules and NK cell recognition, Immunol.Today 11 [7], Seite 237-244. URL: PM:2201309

Lloyd, G.; Bowen, E. T.; Jones, N. und Pendry, A. (1984): HFRS outbreak associated with laboratory rats in UK, Lancet 1 [8387], Seite 1175-1176. URL: PM:6144891

Lloyd, G. und Jones, N. (1986): Infection of laboratory workers with hantavirus acquired from immunocytomas propagated in laboratory rats, J.Infect. 12 [2], Seite 117-125. URL: PM:2871112

Lobigs, M.; Blanden, R. V. und Mullbacher, A. (1996): Flavivirus-induced up-regulation of MHC class I antigens; implications for the induction of CD8+ T-cell-mediated autoimmunity, Immunol.Rev. 152, Seite 5-19. URL: PM:8930665

Lobigs, M.; Mullbacher, A. und Regner, M. (2003): MHC class I up-regulation by flaviviruses: Immune interaction with unknown advantage to host or pathogen, Immunol.Cell Biol. 81 [3], Seite 217-223. URL: PM:12752686

Lu, H. T.; Riley, J. L.; Babcock, G. T.; Huston, M.; Stark, G. R.; Boss, J. M. und Ransohoff, R. M. (1995): Interferon (IFN) beta acts downstream of IFN-gamma-induced class II transactivator messenger RNA accumulation to block major histocompatibility complex class II gene expression and requires the 48-kD DNA-binding protein, ISGF3-gamma, J.Exp.Med. 182 [5], Seite 1517-1525. URL: PM:7595221

Lum, H. und Malik, A. B. (1996): Mechanisms of increased endothelial permeability, Can.J.Physiol.Pharmacol. 74 [7], Seite 787-800. URL: PM:8946065

Lundkvist, Å.; Fatouros, A. und Niklasson, B. (1991): Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies, J.Gen.Virol. 72, Seite 2097-2103. URL: PM:1716651

Lundkvist, Å.; Hörling, J. und Niklasson, B. (1993): The humoral response to Puumala virus infection (nephropathia epidemica) investigated by viral protein specific immunoassays, Arch.Virol. 130 [1-2], Seite 121-130. URL: PM:8099274

Lundkvist, Å.; Meisel, H.; Koletzki, D.; Lankinen, H.; Cifire, F.; Geldmacher, A.; Sibold, C.; Gött, P.; Vaheri, A.; Krüger, D. H. und Ulrich, R. (2002): Mapping of B-cell

epitopes in the nucleocapsid protein of Puumala hantavirus, Viral Immunol. 15 [1], Seite 177-192. URL: PM:11952140

Macatonia, S. E.; Gompels, M.; Pinching, A. J.; Patterson, S. und Knight, S. C. (1992): Antigen-presentation by macrophages but not by dendritic cells in human immunodeficiency virus (HIV) infection, Immunology 75 [4], Seite 576-581. URL: PM:1534309

Mackow, E. R. und Gavrilovskaya, I. N. (2001): Cellular receptors and hantavirus pathogenesis, Curr.Top.Microbiol.Immunol. 256, Seite 91-115. URL: PM:11217408

Makela, S.; Mustonen, J.; Ala-Houhala, I.; Hurme, M.; Partanen, J.; Vapalahti, O.; Vaheri, A. und Pasternack, A. (2002): Human leukocyte antigen-B8-DR3 is a more important risk factor for severe Puumala hantavirus infection than the tumor necrosis factor-alpha(-308) G/A polymorphism, J.Infect.Dis. 186 [6], Seite 843-846. URL: PM:12198621

Marie, I.; Durbin, J. E. und Levy, D. E. (1998): Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7, EMBO J. 17 [22], Seite 6660-6669. URL: PM:9822609

Markotic, A.; Dasic, G.; Gagro, A.; Sabioncello, A.; Rabatic, S.; Kuzman, I.; Zgorelec, R.; Smoljan, I.; Beus, I.; Zupanc, T. A. und Dekaris, D. (1999): Role of peripheral blood mononuclear cell (PBMC) phenotype changes in the pathogenesis of haemorrhagic fever with renal syndrome (HFRS), Clin.Exp.Immunol. 115 [2], Seite 329-334. URL: PM:9933461

Markotic, A.; Hensley, L.; Geisbert, T.; Spik, K. und Schmaljohn, C. (2003): Hantaviruses induce cytopathic effects and apoptosis in continuous human embryonic kidney cells, J.Gen.Virol. 84 [Pt 8], Seite 2197-2202. URL: PM:12867652

Matsukawa, A.; Hogaboam, C. M.; Lukacs, N. W. und Kunkel, S. L. (2000): Chemokines and innate immunity, Rev.Immunogenet. 2 [3], Seite 339-358. URL: PM:11256744

Mayo, D. R. und Beckwith, W. H. (2002): Inactivation of West Nile virus during serologic testing and transport, J.Clin.Microbiol. 40 [8], Seite 3044-3046. URL: PM:12149375

McCaughey, C. und Hart, C. A. (2000): Hantaviruses, J.Med.Microbiol. 49 [7], Seite 587-599. URL: PM:10882083

Miller, D. M.; Zhang, Y.; Rahill, B. M.; Waldman, W. J. und Sedmak, D. D. (1999): Human cytomegalovirus inhibits IFN-alpha-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-alpha signal transduction, J.Immunol. 162 [10], Seite 6107-6113. URL: PM:10229853

Mogensen, T. H. und Paludan, S. R. (2001): Molecular pathways in virus-induced cytokine production, Microbiol.Mol.Biol.Rev. 65 [1], Seite 131-150. URL: PM:11238989

Most, P.; Boerries, M.; Eicher, C.; Schweda, C.; Ehlermann, P.; Pleger, S. T.; Loeffler, E.; Koch, W. J.; Katus, H. A.; Schoenenberger, C. A. und Remppis, A. (2003): Extracellular S100A1 protein inhibits apoptosis in ventricular cardiomyocytes via activation of the extracellular signal-regulated protein kinase 1/2 (ERK1/2), J.Biol.Chem. 278, Seite 48404-48412. URL: PM:12960148

Mustonen, J.; Partanen, J.; Kanerva, M.; Pietila, K.; Vapalahti, O.; Pasternack, A. und Vaheri, A. (1996): Genetic susceptibility to severe course of nephropathia epidemica caused by Puumala hantavirus, Kidney Int. 49 [1], Seite 217-221. URL: PM:8770970

Nagai, T.; Tanishita, O.; Takahashi, Y.; Yamanouchi, T.; Domae, K.; Kondo, K.; Dantas, J. R., Jr.; Takahashi, M. und Yamanishi, K. (1985): Isolation of haemorrhagic fever with renal syndrome virus from leukocytes of rats and virus replication in cultures of rat and human macrophages, J.Gen.Virol. 66, Seite 1271-1278. URL: PM:3925075

Nam, J. H.; Hwang, K. A.; Yu, C. H.; Kang, T. H.; Shin, J. Y.; Choi, W. Y.; Kim, I. B.; Joo, Y. R.; Cho, H. W. und Park, K. Y. (2003): Expression of interferon inducible genes following Hantaan virus infection as a mechanism of resistance in A549 cells, Virus Genes 26 [1], Seite 31-38. URL: PM:12680691

Natarajan, K.; Dimasi, N.; Wang, J.; Mariuzza, R. A. und Margulies, D. H. (2002): Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination, Annu.Rev.Immunol. 20, Seite 853-885. URL: PM:11861620

Navarro, L. und David, M. (1999): p38-dependent activation of interferon regulatory factor-3 by lipopolysaccharide, J.Biol.Chem. 274 [50], Seite 35535-35538. URL: PM:10585427

Navarro, L.; Mowen, K.; Rodems, S.; Weaver, B.; Reich, N.; Spector, D. und David, M. (1998): Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon-stimulated response element-binding complex, Mol.Cell Biol. 18 [7], Seite 3796-3802. URL: PM:9632763

Nichol, S. T.; Ksiazek T.G.; Rollin, P. E. und Peters C.J. (1996): Hantavirus pulmonary syndrome and newly described hantaviruses in the United States, The *Bunyaviridae,* Elliott R.M. (ed.) , Seite 269-280, Plenum Press, New York.

Nichol, S. T.; Spiropoulou, C. F.; Morzunov, S.; Rollin, P. E.; Ksiazek, T. G.; Feldmann, H.; Sanchez, A.; Childs, J.; Zaki, S. und Peters, C. J. (1993): Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness, Science 262 [5135], Seite 914-917. URL: PM:8235615

Nolte, K. B.; Feddersen, R. M.; Foucar, K.; Zaki, S. R.; Koster, F. T.; Madar, D.; Merlin, T. L.; McFeeley, P. J.; Umland, E. T. und Zumwalt, R. E. (1995): Hantavirus pulmonary syndrome in the United States: a pathological description of a disease caused by a new agent, Hum.Pathol. 26 [1], Seite 110-120. URL: PM:7821907

Padula, P. J.; Edelstein, A.; Miguel, S. D.; Lopez, N. M.; Rossi, C. M. und Rabinovich, R. D. (1998): Hantavirus pulmonary syndrome outbreak in Argentina: molecular evidence for person-to-person transmission of Andes virus, Virology 241 [2], Seite 323-330. URL: PM:9499807

Paludan, S. R. (2000): Synergistic action of pro-inflammatory agents: cellular and molecular aspects, J.Leukoc.Biol. 67 [1], Seite 18-25. URL: PM:10647993

Pavlovic, J.; Zurcher, T.; Haller, O. und Staeheli, P. (1990): Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein, J.Virol. 64 [7], Seite 3370-3375. URL: PM:2161946

Pensiero, M. N.; Sharefkin, J. B.; Dieffenbach, C. W. und Hay, J. (1992): Hantaan virus infection of human endothelial cells, J.Virol. 66 [10], Seite 5929-5936. URL: PM:1356160

Peters, C. J.; Simpson, G. L. und Levy, H. (1999): Spectrum of hantavirus infection: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome, Annu.Rev.Med. 50, Seite 531-545. URL: PM:10073292

Pinna, D. M.; Martinez, V. P.; Bellomo, C. M.; Lopez, C. und Padula, P. (2004): Nueva evidencia epidemiologica y molecular a favor de la transmision interhumana para el linaje sout del hantavirus andes, Medicina (B Aires) 64 [1], Seite 43-46. URL: PM:15034956

Platanias, L. C. (2003): The p38 mitogen-activated protein kinase pathway and its role in interferon signaling, Pharmacol.Ther. 98 [2], Seite 129-142. URL: PM:12725866

Plyusnin, A.; Cheng, Y.; Vapalahti, O.; Pejcoch, M.; Unar, J.; Jelinkova, Z.; Lehvaslaiho, H.; Lundkvist, Å. und Vaheri, A. (1995): Genetic variation in Tula hantaviruses: sequence analysis of the S and M segments of strains from Central Europe, Virus Res. 39 [2-3], Seite 237-250. URL: PM:8837887

Plyusnin, A.; Hörling, J.; Kanerva, M.; Mustonen, J.; Cheng, Y.; Partanen, J.; Vapalahti, O.; Kukkonen, S. K.; Niemimaa, J.; Henttonen, H.; Niklasson, B.; Lundkvist, Å. und Vaheri, A. (1997): Puumala hantavirus genome in patients with nephropathia epidemica: correlation of PCR positivity with HLA haplotype and link to viral sequences in local rodents, J.Clin.Microbiol. 35 [5], Seite 1090-1096. URL: PM:9114386

Plyusnin, A.; Vapalahti, O.; Lankinen, H.; Lehvaslaiho, H.; Apekina, N.; Myasnikov, Y.; Kallio-Kokko, H.; Henttonen, H.; Lundkvist, Å.; Brummer-Korvenkontio, M. und . (1994): Tula virus: a newly detected hantavirus carried by European common voles, J.Virol. 68 [12], Seite 7833-7839. URL: PM:7966573

Plyusnin, A.; Vapalahti, O. und Vaheri, A. (1996): Hantaviruses: genome structure, expression and evolution, J.Gen.Virol. 77, Seite 2677-2687. URL: PM:8922460

Poljak, M. und Avsic-Zupanc, T. (1994): Immunohistochemical detection of Hantaan virus antigen in renal tissue from patient with hemorrhagic fever with renal syndrome, Nephron 67 [2], Seite 252. URL: PM:7915405

Powis, S. J.; Townsend, A. R.; Deverson, E. V.; Bastin, J.; Butcher, G. W. und Howard, J. C. (1991): Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter, Nature 354 [6354], Seite 528-531. URL: PM:1758495

Prince, H. N.; Prince, D. L und Prince, R. N (1991): Principles of viral control and transmission, Disinfection, sterilization, and preservation, Block S.S. (ed.), 4th. Auflage, Seite 411-444, Lea & Febiger, Philadelphia.

Raftery, M. J.; Kraus, A. A.; Ulrich, R.; Krüger, D. H. und Schönrich, G. (2002): Hantavirus infection of dendritic cells, J.Virol. 76 [21], Seite 10724-10733. URL: PM:12368315

Raftery, M. J.; Schwab, M.; Eibert, S. M.; Samstag, Y.; Walczak, H. und Schönrich, G. (2001): Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy, Immunity. 15 [6], Seite 997-1009. URL: PM:11754820

Ravkov, E. V. und Compans, R. W. (2001): Hantavirus nucleocapsid protein is expressed as a membrane-associated protein in the perinuclear region, J.Virol. 75 [4], Seite 1808-1815. URL: PM:11160679

Reinhart, K.; Bayer, O.; Brunkhorst, F. und Meisner, M. (2002): Markers of endothelial damage in organ dysfunction and sepsis, Crit.Care Med. 30 [5 Suppl], Seite S302-S312. URL: PM:12004252

Robinson, M. J. und Cobb, M. H. (1997): Mitogen-activated protein kinase pathways, Curr.Opin.Cell Biol. 9 [2], Seite 180-186. URL: PM:9069255

Rosmorduc, O.; Sirma, H.; Soussan, P.; Gordien, E.; Lebon, P.; Horisberger, M.; Brechot, C. und Kremsdorf, D. (1999): Inhibition of interferon-inducible MxA protein expression by hepatitis B virus capsid protein, J.Gen.Virol. 80, Seite 1253-1262. URL: PM:10355772

Samuel, C. E. (2001): Antiviral actions of interferons, Clin.Microbiol.Rev. 14 [4], Seite 778-809. URL: PM:11585785

Schaeffer, H. J. und Weber, M. J. (1999): Mitogen-activated protein kinases: specific messages from ubiquitous messengers, Mol.Cell Biol. 19 [4], Seite 2435-2444. URL: PM:10082509

Schmaljohn, A. L.; Li, D.; Negley, D. L.; Bressler, D. S.; Turell, M. J.; Korch, G. W.; Ascher, M. S. und Schmaljohn, C. S. (1995): Isolation and initial characterization of a newfound hantavirus from California, Virology 206 [2], Seite 963-972. URL: PM:7856107

Schmaljohn, C. S. (1996): Molecular Biology of Hantaviruses., The *Bunyaviridae*, Elliott R.M. (ed.) , Seite 63-90, Plenum Press, New York.

Schmaljohn, C. S. und Hjelle, B. (1997): Hantaviruses: a global disease problem, Emerg.Infect.Dis. 3 [2], Seite 95-104. URL: PM:9204290

Schmaljohn, C. S.; Huggins, J. W. und Calisher, C. H. (1999): Laboratory and field safety., Manual of Hemorrhagic Fever with Renal Syndrome and Hantavirus Pulmonary Syndrome, Lee,H.W.; Calisher,C.H.; Schmaljohn,C.S.(eds.) , Seite 191-198, WHO Collaborating Center for Virus Reference and Research., Seoul.

Schmaljohn, C. S.; Schmaljohn, A. L. und Dalrymple, J. M. (1987): Hantaan virus M RNA: coding strategy, nucleotide sequence, and gene order, Virology 157 [1], Seite 31-39. URL: PM:3103329

Schreiber, R. D. und Farrar, M. A. (1993): The biology and biochemistry of interferon-gamma and its receptor, Gastroenterol.Jpn. 28 Suppl 4, Seite 88-94. URL: PM:8486235

Schultze, D.; Lundkvist, Å.; Blauenstein, U. und Heyman, P. (2002): Tula virus infection associated with fever and exanthema after a wild rodent bite, Eur.J.Clin.Microbiol.Infect.Dis. 21 [4], Seite 304-306. URL: PM:12072943

Sen, G. C. (2001): Viruses and interferons, Annu.Rev.Microbiol. 55, Seite 255-281. URL: PM:11544356

Shen, J.; To, S. S.; Schrieber, L. und King, N. J. (1997): Early E-selectin, VCAM-1, ICAM-1, and late major histocompatibility complex antigen induction on human endothelial cells by flavivirus and comodulation of adhesion molecule expression by immune cytokines, J.Virol. 71 [12], Seite 9323-9332. URL: PM:9371591

Shi, X.; McCaughey, C. und Elliott, R. M. (2003): Genetic characterisation of a Hantavirus isolated from a laboratory-acquired infection, J.Med.Virol. 71 [1], Seite 105-109. URL: PM:12858415

Short, S. M.; Talbott, G. A. und Juliano, R. L. (1998): Integrin-mediated signaling events in human endothelial cells, Mol.Biol.Cell 9 [8], Seite 1969-1980. URL: PM:9693360

Sibold, C.; Meisel, H.; Krüger, D. H.; Labuda, M.; Lysy, J.; Kozuch, O.; Pejcoch, M.; Vaheri, A. und Plyusnin, A. (1999): Recombination in Tula hantavirus evolution: analysis of genetic lineages from Slovakia, J.Virol. 73 [1], Seite 667-675. URL: PM:9847372

Sibold, C.; Ulrich, R.; Labuda, M.; Lundkvist, Å.; Martens, H.; Schütt, M.; Gerke, P.; Leitmeyer, K.; Meisel, H. und Krüger, D. H. (2001): Dobrava hantavirus causes hemorrhagic fever with renal syndrome in central Europe and is carried by two different Apodemus mice species, J.Med.Virol. 63 [2], Seite 158-167. URL: PM:11170053

Smadel J.E. (1953): Epidemic hemorrhagic fever., J.Am.Public Health 43, Seite 1327-1330.

Smith, E. J.; Marie, I.; Prakash, A.; Garcia-Sastre, A. und Levy, D. E. (2001): IRF3 and IRF7 phosphorylation in virus-infected cells does not require double-stranded RNA-dependent protein kinase R or Ikappa B kinase but is blocked by Vaccinia virus E3L protein, J.Biol.Chem. 276 [12], Seite 8951-8957. URL: PM:11124948

Stark, G. R.; Kerr, I. M.; Williams, B. R.; Silverman, R. H. und Schreiber, R. D. (1998): How cells respond to interferons, Annu.Rev.Biochem. 67, Seite 227-264. URL: PM:9759489

Sugimori, T.; Griffith, D. L. und Arnaout, M. A. (1997): Emerging paradigms of integrin ligand binding and activation, Kidney Int. 51 [5], Seite 1454-1462. URL: PM:9150458

Sundstrom, J. B.; McMullan, L. K.; Spiropoulou, C. F.; Hooper, W. C.; Ansari, A. A.; Peters, C. J. und Rollin, P. E. (2001): Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells, J.Virol. 75 [13], Seite 6070-6085. URL: PM:11390609

Suzumura, A.; Lavi, E.; Weiss, S. R. und Silberberg, D. H. (1986): Coronavirus infection induces H-2 antigen expression on oligodendrocytes and astrocytes, Science 232 [4753], Seite 991-993. URL: PM:3010460

Tamura, M.; Asada, H.; Kondo, K.; Takahashi, M. und Yamanishi, K. (1987): Effects of human and murine interferons against hemorrhagic fever with renal syndrome (HFRS) virus (Hantaan virus), Antiviral Res. 8 [4], Seite 171-178. URL: PM:2451468

Taniguchi, T. und Takaoka, A. (2001): A weak signal for strong responses: interferon-alpha/beta revisited, Nat.Rev.Mol.Cell Biol. 2 [5], Seite 378-386. URL: PM:11331912

Temonen, M.; Lankinen, H.; Vapalahti, O.; Ronni, T.; Julkunen, I. und Vaheri, A. (1995): Effect of interferon-alpha and cell differentiation on Puumala virus infection in human monocyte/macrophages, Virology 206 [1], Seite 8-15. URL: PM:7831843

Temonen, M.; Mustonen, J.; Helin, H.; Pasternack, A.; Vaheri, A. und Holthofer, H. (1996): Cytokines, adhesion molecules, and cellular infiltration in nephropathia epidemica kidneys: an immunohistochemical study, Clin.Immunol.Immunopathol. 78 [1], Seite 47-55. URL: PM:8599883

Temonen, M.; Vapalahti, O.; Holthofer, H.; Brummer-Korvenkontio, M.; Vaheri, A. und Lankinen, H. (1993): Susceptibility of human cells to Puumala virus infection, J.Gen.Virol. 74, Seite 515-518. URL: PM:8445370

Thornton, S. C.; Mueller, S. N. und Levine, E. M. (1983): Human endothelial cells: use of heparin in cloning and long-term serial cultivation, Science 222 [4624], Seite 623-625. URL: PM:6635659

Thurston-Enriquez, J. A.; Haas, C. N.; Jacangelo, J.; Riley, K. und Gerba, C. P. (2003): Inactivation of feline calicivirus and adenovirus type 40 by UV radiation, Appl.Environ.Microbiol. 69 [1], Seite 577-582. URL: PM:12514044

Tkachenko, E. A. und Lee, H. W. (1991): Etiology and epidemiology of hemorrhagic fever with renal syndrome, Kidney Int.Suppl. 35, Seite S54-S61. URL: PM:1685201

Tortorella, D.; Gewurz, B. E.; Furman, M. H.; Schust, D. J. und Ploegh, H. L. (2000): Viral subversion of the immune system, Annu.Rev.Immunol. 18, Seite 861-926. URL: PM:10837078

Tulimäki, K.; Kaukinen, P; Weber, F.; Vaheri, A. und and Plyusnin A. (2004): Tula and Puumala hantavirus NSs ORFs are functional., Abstracts 6th International Conference on hantaviruses, Seoul (Korea)., Seite 90.

Van Epps, H. L.; Schmaljohn, C. S. und Ennis, F. A. (1999): Human memory cytotoxic T-lymphocyte (CTL) responses to Hantaan virus infection: identification of virus-specific and cross-reactive CD8(+) CTL epitopes on nucleocapsid protein, J.Virol. 73 [7], Seite 5301-5308. URL: PM:10364276

Van Epps, H. L.; Terajima, M.; Mustonen, J.; Arstila, T. P.; Corey, E. A.; Vaheri, A. und Ennis, F. A. (2002): Long-lived memory T lymphocyte responses after hantavirus infection, J.Exp.Med. 196 [5], Seite 579-588. URL: PM:12208874

Vapalahti, O.; Lundkvist, Å. und Vaheri, A. (2001): Human immune response, host genetics, and severity of disease, Curr.Top.Microbiol.Immunol. 256, Seite 153-169. URL: PM:11217403

Vapalahti, O.; Mustonen, J.; Lundkvist, Å.; Henttonen, H.; Plyusnin, A. und Vaheri, A. (2003): Hantavirus infections in Europe, Lancet Infect.Dis. 3 [10], Seite 653-661. URL: PM:14522264

von Wussow, P.; Jakschies, D.; Hochkeppel, H. K.; Fibich, C.; Penner, L. und Deicher, H. (1990): The human intracellular Mx-homologous protein is specifically induced by type I interferons, Eur.J Immunol. 20 [9], Seite 2015-2019. URL: PM:2120071

Vossen, M. T.; Westerhout, E. M.; Soderberg-Naucler, C. und Wiertz, E. J. (2002): Viral immune evasion: a masterpiece of evolution, Immunogenetics 54 [8], Seite 527-542. URL: PM:12439615

Wathelet, M. G.; Berr, P. M. und Huez, G. A. (1992): Regulation of gene expression by cytokines and virus in human cells lacking the type-I interferon locus, Eur.J.Biochem. 206 [3], Seite 901-910. URL: PM:1318841

Weber, F. und Elliott, R. M. (2002): Antigenic drift, antigenic shift and interferon antagonists: how bunyaviruses counteract the immune system, Virus Res. 88 [1-2], Seite 129-136. URL: PM:12297332

Wells, R. M.; Young, J.; Williams, R. J.; Armstrong, L. R.; Busico, K.; Khan, A. S.; Ksiazek, T. G.; Rollin, P. E.; Zaki, S. R.; Nichol, S. T. und Peters, C. J. (1997):

Hantavirus transmission in the United States, Emerg.Infect.Dis. 3 [3], Seite 361-365. URL: PM:9284382

Wichmann, D.; Grone, H. J.; Frese, M.; Pavlovic, J.; Anheier, B.; Haller, O.; Klenk, H. D. und Feldmann, H. (2002): Hantaan virus infection causes an acute neurological disease that is fatal in adult laboratory mice, J.Virol. 76 [17], Seite 8890-8899. URL: PM:12163608

Williams, B. R. (1991): Transcriptional regulation of interferon-stimulated genes, Eur.J.Biochem. 200 [1], Seite 1-11. URL: PM:1715271

Yanagihara, R. und Silverman, D. J. (1990): Experimental infection of human vascular endothelial cells by pathogenic and nonpathogenic hantaviruses, Arch.Virol. 111 [3-4], Seite 281-286. URL: PM:2112908

Yang, J.; Liu, X.; Bhalla, K.; Kim, C. N.; Ibrado, A. M.; Cai, J.; Peng, T. I.; Jones, D. P. und Wang, X. (1997): Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked, Science 275 [5303], Seite 1129-1132. URL: PM:9027314

Yousefi, S.; Escobar, M. R. und Gouldin, C. W. (1985): A practical cytopathic effect/dye-uptake interferon assay for routine use in the clinical laboratory, Am.J.Clin.Pathol. 83 [6], Seite 735-740. URL: PM:2988328

Zaki, S. R.; Greer, P. W.; Coffield, L. M.; Goldsmith, C. S.; Nolte, K. B.; Foucar, K.; Feddersen, R. M.; Zumwalt, R. E.; Miller, G. L. und Khan, A. S. (1995): Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease, Am.J.Pathol. 146 [3], Seite 552-579. URL: PM:7887439

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Erklärung

Hiermit erkläre ich, dass ich die Dissertation selbst und ohne unzulässige Hilfe Dritter verfaßt habe. Die Arbeit enthält, selbst in Teilen, keine Kopien anderer Arbeiten. Die benutzten Hilfsmittel sowie die Literatur sind vollständig angegeben.

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Publications

Raftery, MJ, Kraus AA, Ulrich R, Krüger DH, and Schönrich G.
Hantavirus infection of dendritic cells.
Journal of Virology. 2002 Nov;76(21):10724-33.

Koch J, Liang M, Queitsch I, Kraus AA, and Bautz EK.
Human recombinant neutralizing antibodies against hantaan virus G2 protein.
Virology. 2003 Mar 30;308(1):64-73.

Kraus AA, Raftery MJ, Giese T, Ulrich R, Zawatzky R, Hippenstiel S, Suttorp N, Krüger DH, and Schönrich G.
Differential antiviral response of endothelial cells after infection with pathogenic and nonpathogenic hantaviruses.
Journal of Virology. 2004 Jun; 78(12): 6143-50

Kraus AA, Priemer C, Heider H, Krüger DH, and Ulrich R.
Inactivation of Hantaan virus-containing samples for subsequent investigations outside biosafety level 3 facilities.
Intervirology, in press (October 2004)

Raftery MJ, Wieland D, Gronewald S, Kraus AA, Giese T, and Schönrich G.
Shaping phenotype, function and survival of dendritic cells by cytomegalovirus-encoded interleukin-10.
Journal of Immunology. 2004 September, 173(5)

Appendix

Table 1. Efficient chemical and physical inactivation or viral removing procedures for HTNV.

Inactivation or depletion procedure	Analysed samples	Titer without treatment [FFU/ml]	Titer after treatment [FFU/ml]	Reduction factor
methanol (absolute) 8 min	lysate	1.5×10^{7}	> 50	> 3×10^{5}
methanol (absolute) 8 min	wash solution	1.5×10^{7}	> 50	> 3×10^{5}
paraformaldehyde (1%) 20 min	lysate	1×10^{6}	> 50	> 2×10^{4}
paraformaldehyde (1%) 20 min	wash solution	1×10^{6}	> 50	> 2×10^{4}
acetone/methanol (1 :1) 10 min	lysate	4×10^{6}	> 10	> 4×10^{5}
detergent-containing lysis buffer 10 min	lysate	5×10^{3}	> 50	> 1×10^{2}
UV-irradiation 1 min	virus solution	1.7×10^{6}	2×10^{2}	8.5×10^{3}
UV-irradiation 3 min	virus solution	1.7×10^{6}	> 10	>1.7×10^{5}
filtration 300 kDa	filtrate	1.5×10^{5}	> 10	> 1.5×10^{4}
filtration 1,000 kDa	filtrate	1.5×10^{5}	1×10^{5}	1.5